Unique and Unifying Themes in the Mechanisms Regulating the Expression of the *Arabidopsis thaliana PR-1* and *Solanum tuberosum PR-10a* inducible defense genes

By

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ABSTRACT

Arabidopsis is a model plant used to study disease resistance; *Solanum tuberosum* or potato is a crop species. Both plants possess inducible defense mechanisms that are deployed upon recognition of pathogen invasion. Transcriptional reprogramming is crucial to the activation of defense responses. The *Pathogenesis-Related* (*PR*) genes are activated in these defense programs. Expression of *Arabidopsis PR-1* and potato *PR-10a* serve as markers for the deployment of defense responses in these plants.

PR-1 expression indicates induction of systemic acquired resistance (SAR). Activation of SAR requires accumulation of salicylic acid (SA), in addition to the interaction of the non-expressor of pathogenesis-related genes 1 (NPR1), with the TGA transcription factors.

The *PR-10a* is activated in response to pathogen invasion, wounding and elicitor treatment. *PR-10a* induction requires recruitment of the Whirly 1 (Why1) activator to the promoter. This locus is also negatively regulated by the silencer element binding factor (SEBF).

We established that both the *PR-1* and *PR-10a* are occupied by repressors under non-inducing conditions. TGA2 was found to be a constitutive resident and repressor of *PR-1*, which mediates repression by forming an oligomeric complex on the promoter. The DNA-binding activity of this oligomer required the TGA2 N-terminus (NT).

Under resting conditions we determined that the *PR-10a* is bound by a repressosome containing SEBF and curiously the activator Pto interacting protein 4 (Pti4). In the context of this repressosome, SEBF is responsible for *PR-10a* binding, yet recruitment of SEBF to this locus required the Pti4.

We also showed that *PR-1* and *PR-10a* are activated by different means. In *PR-1* activation the NPR1 NT domain alleviates TGA2-mediated repression by interacting with the TGA2 NT. TGA2 remains at the *PR-1* but adopts a dimeric conformation and forms an enhanceosome with NPR1. In contrast, the *PR-10a* is activated by evicting the repressosome and recruiting Why1 to the promoter.

These results advance our understanding of the mechanisms regulating *PR-1* and *PR-10a* expression under resting and inducing conditions. This study also revealed that the means of regulation for related genes can differ greatly between model and crop species, which has important implications for the field of translational biology.

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LIST OF ABBREVIATIONS

2,4-D – 2,4-dichlorophenoxyacetic acid

AA – arachidonic acid

AAV – adeno-associated virus

Ac – acidic domain

AP2 – Activator Protein-2

as-1 – activation sequence 1

AtWhy1 – *Arabidopsis thaliana* Why1

Avr – avirulence

BTB/POZ – Broad-Complex, Tramtrack, and Bric-a-brac/Pox virus and Zinc finger

BTH – benzo-1,2,3-thiadiazole-7-carbothioic acid S-methyl ester or benzothiadiazole

bZIP – basic domain/leucine zipper

CaMV – cauliflower mosaic virus

CBP – CREB binding protein

C/EBPβ – CCAAT/Enhancer Binding Protein β

ChIP – chromatin immunoprecipitation

CHX – cycloheximide

cim – constitutive immunity

CK2 – Casein Kinase 2

cs-RBDI – consensus sequence-type RNA binding domain I

cs-RBDII – consensus sequence-type RNA binding domain II

Ct – threshold cycle

cv - cultivar

Cys – cysteine

DB – DNA binding domain

DDM – DNA demethylase

DTT – dithiothrietol

E1A – early 1A

EMSA – electrophoretic mobility shift assay

ERE –elicitor response element

ERF – ethylene response factor

FCA – fluorescence complementation assay

GAL – galactosidase

Gal – Galactose

GAL4 DB – GAL4 DNA-binding domain

GFP – green fluorescent protein

Glu – Glucose

Gly – Glycine

GST – glutathione S-transferase

GTF – general transcription factors

H3 – histone 3

H4 – histone 4

HDAC – histone deacetylase

HDM – histone demethylase

HA – hemagglutinin

HAT – histone acetyltransferases

His – Histidine

HMT – histone methyltransferase

hnRNP – heterogeneous nuclear ribonucleoprotein

HP1 – heterochromatin protein 1

HR – hypersensitive response

IEGT1 – immediate-early-induced glucosyl transferase 1

INA – 2,6-dichloroisonicotinic acid

IP – immunoprecipitation

JA – jasmonic acid

kD(a) – kilodalton

kd – dissociation constant

LacZ -

Leu – leucine

Kr – Krüppel

LS – linker-scanning

lsd – lesion simulating disease

MAP – mitogen-activated protein

MBP – methylated DNA binding protein

MeJA – methyl jasmonate

MeSA – methyl salicylate

MPB – 3-(N-maleimido-propionyl) biocytin

MYB – myeloblastosis

NADPH – nicotinamide adenine dinucleotide phosphate

N-coR – Nuclear receptor-corepressor

NEM – N-ethylmaleimide

NES – nuclear export sequence

NIM1 – non-inducible immunity 1

NLS – nuclear localization sequence

nos – nopaline synthase

NPR1 – non-expressor of pathogenesis related genes 1

NR – Nuclear receptor

NT – N-terminus

ONPG – ortho-nitrophenol-b-D-galactopyranoside

PAGE – polyacrylamide gel electrophoresis

PB – PBF-2 binding element

PBF-2 – PR-10a binding factor 2

PCR – polymerase chain reaction

PDF1.2 – plant defensin 1.2

PIC – Pre-initiation complex

PR – Pathogenesis related

Pti4 – Pto interacting protein 4

Pto – *Pseudomonas syringae* pv. *tomato* resistance gene

PTM – post-translation modification

pv – pathovar

qPCR – quantitative PCR

R – resistance

RNAi – RNA interference

RNAPII – RNA polymerase II

SA – salicylic acid

SAA1 – serum amyloid A1

SAI1 – salicylic acid insensitive 1

SAR – systemic acquired resistance

SDS – sodium dodecyl sulfate

SE – silencer element

SEBF – SE binding factor

SMRT – silencing mediator for retinoid and thyroid receptors

SNI1 – suppressor of *npr1-1*, inducible 1

Sp3 – specificity protein 3

StWhy1 – *Solanum tuberosum* Why1

ssDNA – single-stranded DNA

SUMO – small ubiquitin-like modifier

SWI/SNF – Sucrose non fermentation/Mating type switching

TA – transactivation domain

TATA – TATA box

t-CA – trans cinnamic acid

TCF/LEF – leukocyte enhancer factor/T-cell factor

TF – transcription factor

TMV – tobacco mosaic virus

Trp – Tryptophan

TSS – transcriptional start site

UAS – upstream activation sequence

VIGS – virus induced gene silencing

Vir – virulence

VP16 – viral particle 16

Why1 – Whirly 1

X-Gal – 5-bromo-4-chloro-3-indoyl-b-D-galactopyransoide

YY1 – Yin Yang 1

CHAPTER 1 – INTRODUCTION

Plants have evolved an immune system that enables them to deploy an array of defense mechanisms upon perception of pathogen invasion (Durrant and Dong, 2004). Crucial to the induction of these colonization counter measures is the rapid activation of a battery of defense genes, including the *PR* genes (Jones and Dangl, 2006). While numerous studies have focused on identifying those genes up-regulated in response to pathogen attack (Schenk et al., 2000; Reymond et al., 2000; Maleck et al., 2000; Eulgem, 2005), the mechanisms that govern the expression of inducible defense genes remain largely unknown. Investigating the agents and elements orchestrating *PR* gene expression at the promoter, in both the model system *Arabidopsis* and the crop species potato, will serve to further our understanding of the molecular mechanisms by which these plants activate the transcriptional reprogramming critical to the combat of disease. This research approach will also provide valuable insights as to the degree of conservation in the mechanisms of defense gene regulation between a model plant and a crop species. The knowledge garnered from such research can be used towards the development of durable and environmentally friendly disease resistance in crop species.

1.1 Outline

The objective of the research presented in this thesis is to investigate the unifying themes and unique features in the means by which the combinatorial interactions of DNA regulatory elements, transcription factors and cofactors coordinate the regulation of the *Arabidopsis PR-1* and potato *PR-10a* inducible defense-related genes. This research has

broad implications for the ability to translate the findings from the study of *Arabidopsis*, a model plant, into potato, a crop species.

Chapter 2 is a literature review, which examines how the interplay of cis- and trans-acting elements at the promoter manifests specific transcriptional states, with particular emphasis on dual function transcription factors. This chapter also provides a comprehensive review of the means by which trans- and cis-acting elements combine to regulate expression of the *Arabidopsis PR-1* and potato *PR-10a* inducible defense genes.

Chapter 3 is a published manuscript which demonstrates that the TGA2 transcription factor and NPR1 coactivator occupy the promoter of the *Arabidopsis PR-1* gene constitutively and that these regulators are recruited to this locus independently. These findings establish that the TGA2 functions as a dual function transcription factor, required for *PR-1* repression under resting conditions yet is essential to the induction of *PR-1* expression following salicylic acid (SA) stimulation. Finally, functional dissection of the NPR1 identified a novel transactivation domain, termed the cysteines (Cys)-oxidized motif, which is required for coactivator function.

Chapter 4 is a published manuscript that addresses the composition of the SE binding factor (SEBF) repressosome complex at the potato *PR-10a* promoter. Interestingly Pti4, a known transcriptional activator, is shown to be an essential component of the transcriptional repressor complex. This discovery establishes that Pti4 is a dual function factor. Chromatin immunopreciptation (ChIP) experiments also suggest that the mechanism for *PR-10a* activation proceeds through an initial eviction of the SEBF-Pti4 repressosome from the promoter and subsequent recruitment of the Whirly 1 (Why1) transcriptional activator.

Chapter 5 is a submitted manuscript that specifically identifies a structural basis for TGA2-mediated repression of the *PR-1*, using both in vitro and in vivo methods. The findings also demonstrate the NPR1 Broad-Complex, Tramtrack, and Bric-a-brac/Pox virus and Zinc finger (BTB/POZ) domain is specifically responsible for the alleviation of TGA2-dependent *PR-1* repression. This conclusion is again supported by the convergence of in vitro and *in planta* data. Collectively, the findings presented in this manuscript serve to further our mechanistic understanding of the regulation of *PR-1* expression under both resting and inducing conditions.

Chapter 6 discusses the common and contrasting themes in the mechanisms regulating the expression of *PR-1* and *PR-10a*. This chapter also proposes some future experiments to address some unappreciated and uninvestigated aspects of the *PR-1* and *PR-10a* regulatory apparatus.

CHAPTER 2 – LITERATURE REVIEW – GENE REGULATION AND INDUCIBLE PLANT DEFENSE RESPONSES

2.1 Transcriptional reprogramming is an essential element of plant inducible defense responses

Plants are armed with an arsenal of inducible defensive mechanisms that can be deployed to combat microbial colonization. The activation of these defenses entails the stimulation of a network of signal transduction pathways and large-scale gene induction (Jones and Dangl, 2006). The perception of pathogen invasion by a plant cell triggers numerous signals that are transduced to the nucleus where they are processed by various transcription factors, resulting in the coordinated activation of a massive array of defense related genes (Desveaux et al., 2005). Studies have shown that as much as one quarter of all the genes in the model plant *Arabidopsis* demonstrate altered expression in response to pathogen attack (Eulgem, 2005).

2.2 Opening the locked door to gene activation: the separate states of transcription

Central to gene regulation is the ability to manifest, maintain, and modulate distinct transcriptional states. The eukaryotic promoter serves as a doorway for the basal transcription machinery, enabling this apparatus to access the transcription start site, which is a prerequisite for gene expression. Thus the state of gene activation is defined largely by the status of this doorway (Figure 1).

All promoters, when present as a naked DNA template in the company of the basal transcriptional machinery, demonstrate an inherent level of gene activity referred to as the basal level of transcription (Roeder, 2005). The doorway in this case is open and therefore permissive to transcription. The extent to which the door is open will vary considerably as a function of the DNA sequence present at the promoter (Struhl, 1999). However, it should be understood that this basal level of activation is generally not observed in eukaryotic systems because chromatin structures impose a non-permissive transcriptional ground state (Struhl, 1999; Roeder, 2005; Heintzman and Ren, 2007; Li et al., 2007). Chromatin effectively slams the door shut on the basal transcription apparatus by rendering cis-elements such as the TATA box, required for the recruitment of this machinery, inaccessible.

The chromatinized promoter can be viewed as a closed door, fastened shut with a bolt, which defines the ground state of eukaryotic gene activation. Just as an already closed door cannot be closed any further, there is considerable difficulty in demonstrating that promoter transcriptional output can exist below this ground state using in vitro transcription systems. However, this does not preclude the possibility of further negative gene regulation or repression. A closed door cannot be further closed but it can be fortified in this closed position through the introduction of various locks. Further states of repression are achieved through the recruitment of sequence-specific DNA-binding transcription factors, known as repressors, to the promoter through cis-regulatory elements.

It should be noted that while even the simplest of chromatin templates are sufficient to occlude the recruitment of the basal transcriptional machinery, nucleosomes

present a relatively modest barrier to the DNA-binding activity of transcription factors (Struhl, 1999; Li et al., 2007). Upon binding to regulatory elements in the proximal promoter region, repressors are able to recruit corepressor complexes that possess a multitude of chromatin-modifying activities. Some of these activities are aimed at specifically antagonizing histone modifications associated with gene activation, while others work to recruit further repressive entities to the locus (Rosenfeld et al., 2006). Corepressor complexes also include a family of ATP-dependent nucleosome-remodeling factors that function to further constrain chromatin structures (Rosenfeld et al., 2006). It is important to note that these multisubunit corepressor complexes can be recruited in a parallel and/or sequential manner (Rosenfeld et al., 2006). The histones present in the promoters of poised and active genes are typically acetylated and phosphorylated at key residues. Corepressors commonly boast histone deacetylase (HDAC) and phosphatase activities that remove these activating marks (Roeder, 2005; Rosenfeld et al., 2006; Heintzman and Ren, 2007; Li et al., 2007). Other chromatin modification activities include those mediated by corepressors such as histone methyltransferases (HMTs) and histone demethylases (HDMs), which conjugate and remove methyl moieties from histone tails, respectively (Rosenfeld et al., 2006; Garcia-Bassets et al., 2007). Methylation of histone H3 lysines at positions 9 (H3K9) and 27 (H3K27) as well as histone H4 lysine 20 (H4K20) are associated with repression (Rosenfeld et al., 2006; Li et al., 2007), while methylation at H3K4 is commonly observed at the promoters of activated genes (Rosenfeld et al., 2006; Garcia-Bassets et al., 2007; Li et al., 2007). Activities that methylate H3K9, H3K27 and H4K20 and those which demethylate H3K4 are featured among those present in corepressor complexes (Rosenfeld et al., 2006;

Garcia-Bassets et al., 2007). Ultimately, these events occlude the recruitment of any coactivators, securing the promoter in a non-permissive state. Furthermore, they can also serve to direct the recruitment of entities that can manifest the most severely constrained and repressed chromatin structure, known as facultative heterochromatin (Rosenfeld et al., 2006).

A heterochromatinized promoter is a doorway sealed shut. In this state the gene is no longer competent for activation and it is deemed transcriptionally silent. The term transcriptional silencing is rather ambiguous because it is currently employed to define a number of related yet different phenomena. When used in a strictly transcriptional context, a silent gene refers to a repressed or inactive gene. The terms "silenced" and "repressed" are essentially interchangeable. In the field of epigenetics, gene silencing carries a distinct connotation in that it refers to a maintained and heritable state of gene repression, which is effected through the facultative heterochromatinization of the loci (Hsieh and Fischer, 2005). In this article, we will be using the term "silencing" in the epigenetic sense because it appreciates the greater state of repression that is imposed by the heterochromatin structure. From the perspective of the RNA polymerase, a heterochromatinized promoter presents a far greater barrier than that of an actively repressed promoter despite the fact that both are transcriptionally inactive, much like a doorway sealed shut is considerably more difficult to open than a locked door, even though both doorways are equally closed.

The heterochromatinization of a gene is manifested through a number of characteristic modifications at the promoter, most notably DNA cytosine methylation, primarily but not exclusively in the context of CpG dinucleotides, and histone

methylation at position H3K9 (Mutskov and Felsenfeld, 2004; Naumann et al., 2005; Stancheva, 2005). These modifications demonstrate a puzzling interdependence; however, they clearly both contribute to the establishment of transcriptionally silent heterochromatinized loci (Stancheva, 2005). Beyond the interdependence of DNA methylation and H3K9 methylation, these modifications also serve in the recruitment of distinct protein entities. DNA methylation enables the recruitment of methylated DNA binding proteins (MBPs), while the H3K9 methylation mark is responsible for directing the heterochromatin protein-1 (HP1; in plants the HP1 homolog is known as like-HP1 or LHP1; Gaudin et al., 2001) to the locus. These entities essentially function to constrict and compact the chromatin into the conformation known as heterochromatin.

Opening a locked door requires three separate steps: 1) unlocking the door; 2) turning the knob to release the bolt from the latch; and 3) finally opening the door. The activation of an actively repressed gene proceeds through a similar three-step procedure. In order to unlock a gene from a repressed state, it is necessary to alleviate the repressive chromatin modifications and structures at the promoter. Accomplishing this feat typically entails the dismissal of repressive transcription factors, allowing for the subsequent recruitment of activator(s), which are other sequence-specific DNA-binding factors that bind cis-elements present in the proximal promoter. There are also a number of cases in which the repressor is converted into an activator through the binding of a ligand or via a post-translational modification (Rosenfeld et al., 2006).

Activators function to recruit coactivator complexes to the promoter, and much like their antagonists, the corepressors, these complexes can be placed into two distinct classes: those which serve to recruit and stabilize the transcriptional apparatus and those

that effect the remodeling and modification of the chromatin (Roeder, 2005; Rosenfeld et al., 2006). Members of the first class of coactivators are often referred to as adaptors. These entities form a direct bridge between the activator and the basal transcriptional machinery. The most notable example of an adaptor is the multisubunit mediator complex (Roeder, 2005). The mediator is conserved among most eukaryotic organisms and is a necessary component for activator-driven transcription (Roeder, 2005). Not only do the adaptor coactivators, such as the mediator, direct the recruitment of the general transcription factors and RNA polymerase II (RNAPII) to the promoter, but they also provide a means to communicate regulatory information from the activator and cis-regulatory elements to the transcription machinery (Roeder, 2005).

The second class of coactivator complexes, which target their activities to the chromatin, are typically grouped into two subclasses; the histone modifiers and the remodelers. The histone modifier class boasts the ability to perform a myriad of post-translational modifications, including acetylation, methylation, demethylation, phosphorylation, ubiquitylation, sumoylation, and Poly(ADP-ribosyl)ation, most of which are targeted to the N-terminal tails of histones H3 and H4 in the nucleosome (Rosenfeld et al., 2006). Histone acetyltransferases (HATs) constitute a major component of coactivator complexes. Promoter histone hyperacetylation is a common feature of active genes (Rosenfeld et al., 2006; Rando and Ahmad, 2007). This modification is proposed to facilitate gene activation by three different mechanisms. First of all, the introduction of acetyl moieties alters the net charge of nucleosomes, attenuating DNA-histone interactions and ultimately rendering nucleosomes easier to displace (Li et al., 2007). Secondly, acetylation of the H4K16 position has also been shown to prevent the

formation of compact higher-order chromatin structures (Li et al., 2007). Finally, histone acetylation provides distinct marks, or tags, that permit the recruitment of other proteins to the locus, which can facilitate various aspects of derepression and gene activation (Rosenfeld et al., 2006; Heintzman and Ren, 2007; Li et al., 2007). The activities associated with these coactivators are also responsible for the modifications of components of the transcriptional machinery, and such modifications control critical events in transcriptional regulation (Rosenfeld et al., 2006).

The second subclass of chromatin-directed coactivators, the histone remodelers, employs components of the ATP-dependent chromatin-remodeling machinery. This group includes entities such as the SWI/SNF (Sucrose non fermentation/Mating type switching) complex, which can compromise histone-DNA interactions in the nucleosome, enabling nucleosome sliding and eviction (Rosenfeld et al., 2006; Li et al., 2007; Rando and Ahmad, 2007). Such activities are essential to the displacement of nucleosomes from the TATA box, freeing this important cis-element for binding by the general transcription machinery (Li et al., 2007; Rando and Ahmad, 2007). The remodeling machinery is also involved in histone replacement and the installment of histone variants such as H3.3 and H2A.Z, both of which are enriched in promoter regions (Li et al., 2007; Rando and Ahmad, 2007). The presence of these variants is believed to primarily influence local chromatin architecture, rather than affecting the histone code-driven recruitment of ancillary factors, because the variants differ very little with respect to the sites of modification in canonical histones (Li et al., 2007). It should be noted that various coactivator complexes can be recruited in parallel. However, some of these chromatin-modifying activities are required to take place first, before the recruitment of

subsequent coactivators and adaptors (Struhl, 1999; Rosenfeld et al., 2006). The collective efforts of the various coactivators provide a means to unlock a repressed promoter from its restricted state.

In order to open an unlocked door, you need only to turn the knob and open it. However, turning the knob is a mechanistically, and possibly temporally, distinct step from opening the door. The restructuring at the promoter, mediated by the coactivators, permits binding of the general transcription factors and RNAPII, giving rise to what is known as the pre-initiation complex (PIC) (Roeder, 2005; Heintzman and Ren, 2007). The assembly of the PIC renders a gene poised for activation. The mediator complex, which makes direct contact with aspects of the general transcription factors and RNAPII, plays a key role in regulating the initiation of transcription from the PIC, poised at the promoter (Roeder, 2005; Heintzman and Ren, 2007). This poised state is comparable to standing in front of a door with the knob turned and the bolt completely removed from the latch.

The final act of opening the door to gene activation begins with melting of the DNA around the transcription start site, allowing RNAPII access to the template strand, and from this point, transcription proceeds. It should be noted that activated transcription far exceeds the level of gene activity produced from the naked template and the basal transcription machinery (Roeder, 2005). The collective efforts of the activators and coactivators not only alleviate the restrictive state imposed by the chromatin and repressors, but also serve to establish an environment for the optimal performance of the transcription apparatus (Roeder, 2005).

Passing through the doorway to gene activation is a complicated matter in eukaryotes because of the locked door imposed by chromatin and repressors. However, the system boasts an array of activities that can perform the separate acts of unlocking the door, releasing the latch, and opening it up wide.

2.3 Distinguishing duality among treasonous transcription factors

According to the conventional wisdom on transcription factors, activators recruit coactivators, resulting in gene activation, while repressors recruit corepressors, resulting in the repression of transcription. However, there are a great number of cases in which a transcription factor that activates and recruits coactivators in one instance can recruit corepressors in another (Latchman, 2001; Ma, 2005; Rosenfeld et al., 2006). The term "dual function" is assigned to many transcription factors based entirely upon their ability to mediate both gene activation and repression events. However, upon investigating the conditions under which this duality is demonstrated, it becomes apparent that there are different classes of dual-function factors. The treasonous behavior of these transcription factors is typically demonstrated in a context- or signal-dependent manner (Latchman, 2001; Ma, 2005)

2.3.1 Context dependent duality

The ability of a transcription factor to selectively recruit a coactivator or corepressor is not a purely intrinsic property, and is often shaped by the DNA sequence to which the

factor is bound, the structure of the surrounding chromatin, and the type of molecules available in the nuclear milieu.

The dual nature of many transcription factors is promoter-dependent. In these cases, a factor acts as an activator in the context of one promoter but represses in the context of another. The basis for this differential recruitment is attributed to differences in the DNA sequence of the cis-regulatory elements occupied by the factor (Latchman, 2001; Natoli, 2004; Ma, 2005; Rosenfeld et al., 2006; Heintzman and Ren, 2007). Transcription factors tend to tolerate some amount of sequence variation in their cognate binding elements, as evidenced by their general ability to bind several degenerate sequences with a high affinity (Latchman, 2001; Natoli, 2004; Heintzman and Ren, 2007). The ability of these factors to recognize degenerate target sequences is central to their capacity to recruit different cofactors. One often neglects to consider the contributions of cis-regulatory elements in gene regulation. The DNA sequences in regulatory elements are much more than simply an address in the genome that is to be recognized by a specific transcription factor. DNA binding can produce drastic changes in transcription factor conformation (Natoli, 2004). The transcription factor DNA-binding (DB) domains will adopt different conformations in order to optimize interactions with a cis-element, and therefore different DNA sequences will have different conformational consequences (Natoli, 2004). The conformation adopted by the factor in response to DNA binding will ultimately influence the positioning and accessibility of cofactor interaction motifs in the transcription factor complex (Latchman, 2001; Natoli, 2004; Ma, 2005; Rosenfeld et al., 2006; Heintzman and Ren, 2007). In essence, cis-elements aid in sculpting the structures and surfaces being broadcasted by DNA-bound transcription

factors into the cellular milieu, directly influencing which cofactors will be recruited to the locus. This phenomenon is evidenced by the work of Leung et al. (2004) in which it was demonstrated that a single nucleotide mutation in the binding site for the NF-κB transcription factor results in the recruitment of a coactivator complex different from the one normally recruited when NF-κB is bound to the unmodified promoter element. A true example of promoter-dependent transcription factor duality is demonstrated by the glucocorticoid receptor (GR). This Nuclear Receptor (NR) transcription factor only binds its cis-regulatory elements in response to treatment with the corresponding hormone (Latchman, 2001). The steroid-bound transcription factor binds two different cis-elements termed GRE (glucocorticoid response element) and nGRE (negative GRE). With the former, the factor binds the element as a dimer, which results in gene activation. However, GR binds the latter as a trimer and this entity represses gene expression.

A number of factors are reported to demonstrate cell- or tissue-dependent dual activator/repressor function. However, these opposing behaviors are often manifested on different cis-elements and therefore, technically, constitute examples of promoter-dependent duality. That being said, there are also instances in which the capacity of a transcription factor to activate or repress a given promoter is dictated in an entirely cell-or tissue-dependent manner. The mammalian HES-1 factor demonstrates cell type-dependent dual function. This basic helix-loop-helix (bHLH) transcription factor acts as a repressor of the human acid α-glucosidase (GAA) gene through a 25-bp silencer element in Hep G2 cells. However, this same promoter element was found to function as an enhancer in human fibroblast cells. The level of gene activation was increased as a result of over-expressing the HES-1 factor, while deletion of the HES-1 binding site in the

GAA 25-bp promoter element abrogated gene activation (Yan et al., 2002). The Pit-1 is a tissue-specific transcription factor, which demonstrates both promoter- and cell-dependent dual activator/repressor functions. The factor is required to activate the expression of *growth hormone 1* (*GH1*) in one somatotrope cell type, yet acts to repress *GH1* expression in lactotrope cells (Scully et al., 2000).

Regulation of cell- or tissue-specific genes is often governed by cell type-specific transcription factors and cofactors (Ren and Liao, 2001; Hochheimer and Tjian, 2003; Taatjes et al., 2004). The ability of a transcription factor to function as an activator or repressor can be entirely the consequence of the unique complement of factors and cofactors expressed in a particular cell type (Ren and Liao, 2001; Ma, 2005). The tissue specificity of transcription factors can be manifested through competitions among these factors for certain cis-regulatory elements and cofactors in the target tissue type. The AP-2 (Activator Protein-2) is so-named for its ability to activate transcription. However, this factor is necessary for the repression of the *Serum Amyloid A1* (*SAA1*) gene in non-hepatic cells. The activation of the *SAA1* gene requires the transcription factor NF-κB. In this case, the NF-κB-binding site overlaps with that of the AP-2 in the *SAA1* promoter. Protein binding experiments demonstrated that the interaction of AP-2 or NF-κB with this overlapping binding site is mutually exclusive (Ren and Liao, 2001). It was also shown that the ability to repress the *SAA1* promoter activation in HeLa cells was contingent upon the presence of the AP-2-binding element (Ren and Liao, 2001). In this situation, a tissue-specific transcription factor, AP-2, serves to prevent the aberrant expression of a liver-specific gene in non-hepatic cells by displacing the activator NF-κB from its enhancer element. The prototypical dual-function transcription factor YY1 (Yin Yang 1)

is proposed to mediate the repression of some genes by way of a very similar mechanism. However, this is only one of many means by which this factor can negatively regulate gene expression (Ma, 2005; Gordon et al., 2006).

The competition among transcription factors extends to cofactors. The availability of these cofactors can be a key determinant of transcription factor behavior (Ma, 2005). This concept of limiting concentrations of coactivators affecting gene regulation programs is based largely on what is observed in the Rubenstein-Taybi syndrome (Rosenfeld et al., 2006). This disorder, which is characterized by severe development abnormalities, arises as a result of haplo-insufficiency of the ubiquitous coactivator CBP (CREB binding protein, a.k.a. p300), meaning that only half of the normal amount of this HAT-containing coactivator is present in the cells. Further supporting the notion that cofactor concentration can dictate transcription factor function can be found in the Wnt signaling pathway. Typically, following activation of the canonical Wnt pathway, the β-catenin coactivator is translocated from the cytosol to the nucleus (Kikuchi et al., 2006). In the nucleus, β-catenin interacts with the TCF/LEF (leukocyte enhancer factor/T-cell factor) transcription factor, forming a transactivating complex that activates the expression of a number of genes (Kikuchi et al., 2006). However, when non-TCF/LEF transcription factors are present at high concentrations, they can compete for interaction with β-catenin, yielding a very different transcriptional program (Rosenfeld et al., 2006).

Another example of how cofactor availability can dictate the function of a dual-acting transcription factor can be seen in the regulation of the adeno-associated virus (AAV) P5 promoter by the YY1 factor. As previously mentioned, YY1 is the prototypical dual-function transcription factor, and in this case, it mediates repression of

AAV P5. However, coinfection with adenovirus results in the production of the adenovirus coactivator Early 1A (E1A) (Chang et al., 1989). The E1A coactivator is recruited to the AAV P5 promoter in an YY1-dependent manner. The E1A and YY1 collectively recruit the p300 HAT coactivator complex, resulting in the activation of the AAV P5 locus. YY1 is known to exert transcriptional activation and repression through a number of different mechanisms and to mediate interactions with both HAT and HDAC cofactors (reviewed in Thomas and Seto, 1999; Gordon et al., 2006). The means by which the E1A is able to convert YY1 from a repressor to an activator is unclear. However, it has been proposed that the interaction with E1A elicits a conformational change in the transcription factor that masks the repression motif while unveiling concealed activation domains (Gordon et al., 2006).

The concentration of a transcription factor itself can also govern if the factor will function as an activator or a repressor at a given promoter. The Krüppel (Kr) zinc finger protein is an example of such a transcription factor. At low concentrations, Kr binds DNA as a monomer, which activates transcription. However, at high concentrations, the transcription factor forms a homodimer, which binds the same DNA sequence as the monomeric species, but functions exclusively as a repressor (Sauer and Jackle, 1993; Sauer et al., 1995).

Many transcription factors boast dual functions. However, the ability of a transcription factor to affect gene activation or repression is rarely inherent to the factor and is most often owed to its environment, as defined by the regulatory elements upon which it sits, the other DNA-binding factors that surround it, and the constellation of cofactors available to it.

2.3.2 Signal-dependent duality

The ability of a dual-acting transcription factor to switch from a repressor to an activator or vice versa can be regulated in a signal-dependent manner. This behavior is clearly demonstrated by the NR family of transcription factors. The ability of these factors to recruit HAT coactivators is typically contingent upon their binding of a ligand (Ma, 2005; Rosenfeld et al., 2006). The ligands include a number of steroids and hormone species (Ma, 2005). In the absence of their cognate ligands, the NR transcription factors mediate the recruitment of HDAC corepressor complexes through interactions with the Nuclear Receptor-coRepressor (N-coR) and Silencing Mediator for Retinoid and Thyroid Receptors (SMRT) components (Rosenfeld et al., 2006; Ma, 2005). The differential recruitment of cofactors mediated by the ligand-bound and unbound species is attributed to conformational changes in the NR-cofactor interaction interface induced by ligand binding (Ma, 2005).

Plants do not possess NR transcription factors. However, the duality of many other classes of eukaryotic transcription factors is also regulated in a signal-dependent manner, but not as directly as that observed with NR factors. Signal transduction pathways often result in the post-translational modification of transcription factors. Modifications, such as phosphorylation and sumoylation, can effect the conversion of repressor to activator and activator to repressor, respectively (Ma, 2005). For example, the CCAAT/Enhancer Binding Protein β (C/EBPβ), a basic leucine-zipper (bZIP) transcription factor, is a component of the Ras signal transduction pathway. C/EBPβ is converted from a transcriptional repressor to activator following Ras-dependent

phosphorylation (Mo et al., 2004). It is important to note that both the repressor and activator functions of this factor are exerted at the same locus through the same binding site in the promoter (Mo et al., 2004). Both the repressive and activating forms of C/EBPβ recruit the Mediator adaptor complex. However, following Ras-dependent phosphorylation of the transcription factor, a component of the Mediator, the Trap230/Trap240/CDK8/cyclinC subcomplex, known to be recruited to repressed genes, was absent from the complex (Conaway et al., 2005). The phosphorylated and unphosphorylated forms of C/EBPβ interact with different subunits of the Mediator (Mo et al., 2004). It is proposed that the conformation adopted by the Mediator complex, in the presence of the phosphorylated C/EBPβ, destabilizes the interaction between the Mediator core subunits and the Trap230/Trap240/CDK8/cyclinC subcomplex, resulting in the detachment of this repressive component (Mo et al., 2004).

The ability of the Sp3 (Specificity Protein 3) zinc finger transcription factor to act as either a repressor or an activator is contingent upon an interplay between sumoylation and acetylation (Valin and Gill, 2007). Sp3 must be sumoylated in order to function as a repressor, while acetylation is required for strong activation (Valin and Gill, 2007). SUMO (Small Ubiquitin-Like Modifier) is a 101-amino acid peptide that is conjugated to a lysine residue in the target protein through a process similar to ubiquitylation (Verger et al., 2003). Notably, the expression of SUMO as a translational fusion with the GAL4 DB is sufficient to repress transcription in reporter gene assays (Verger et al., 2003). This modification is proposed to serve as a platform that aids in the recruitment of HDAC-containing corepressors. However, there is also evidence for HDAC-independent SUMO-mediated repression (Valin and Gill, 2007).

The signal-dependent class of dual-acting transcription factors function as molecular sensors, enabling the modulation of transcription programs in response to various stimuli. Essential to performing this role is the conformational diversity that these factors boast, a potential that is bolstered by their ability to accommodate various types and combinations of post-translational modifications. These modifications serve to further diversify the interaction and recruitment motifs offered by the transcription factors.

Contrary to conventional beliefs, not all transcription factors behave as agents that mechanically bind a DNA sequence and recruit coactivators or corepressors based simply on their exclusive nature as either activator or repressor. While there are some examples of transcription factors that go about ignorantly imposing their function upon a gene, there are also factors that formulate their function as a result of their environment as well as others that serve as molecular sensors that can switch functions in response to a single signal. It is the collective action of these various classes of factors that coordinate the diverse yet precise transcriptional programs in response to complex stimuli.

2.4 Arabidopsis *PR-1* gene expression is the molecular marker for the induction of systemic acquired resistance (SAR)

SAR is an inducible defense mechanism that is deployed in response to local pathogen attack producing a long lasting heightened state of disease resistance throughout the plant (Grant and Lamb, 2006; van den Burg and Takken, 2009). Activation of SAR involves global transcription reprogramming (van den Burg and Takken, 2009). Among the genes

up-regulated in this systemic defense response are a suite of *PR* genes, including the *PR-1* (Grant and Lamb, 2006). A necessary prerequisite for the establishment of SAR and *PR-1* gene activation is the accumulation of the endogenous signaling molecule salicylic acid (SA) (van den Burg and Takken, 2009). Exogenous application of SA or its chemical analogs, 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole are also sufficient for *PR-1* gene activation and the deployment of SAR, in a process referred to as chemical SAR (Oostendorp et al., 2001).

PR-1 gene expression is orchestrated through the concerted efforts of the transcriptional regulator non-expressor of pathogenesis related genes 1 (NPR1), the TGA2-containing clade of transcription factors, and cis-regulatory elements residing in the *PR-1* promoter region (Lebel et al., 1998; Zhang, et al., 2003; Durrant and Dong 2004).

The NPR1 protein is recognized as the master positive regulator of SAR (Cao et al., 1994; Durrant and Dong, 2004). Mutations in the *NPR1* gene compromise SA-dependent transcriptional reprogramming leaving the plant unable to mount an effective SAR response (Cao et al., 1994). The *NPR1* gene is constitutively expressed, however the expression is up-regulated a modest two-fold in response to SA (Cao et al., 1998). Overexpression of *NPR1* confers enhanced disease resistance but does not result in constitutive expression of the *PR-1* (Cao and Dong, 1998). The heightened state of defense observed in the *NPR1* overexpressing plants is attributed to increased induction of the *PR* genes following stimulation with SA (Cao and Dong, 1998). These findings indicate that the NPR1 protein is likely to undergo an SA-dependent modification in order to induce transcriptional reprogramming. Another factor shown to function in the

SA-dependent activation of *PR-1* is the *Arabidopsis thaliana* Whirly 1 (AtWhy1) transcriptional activator (Desveaux et al., 2004). It is understood that the AtWhy1 operates through an NPR1-independent pathway but the involvement of this factor in *PR-1* regulation remains largely uninvestigated (Desveaux et al., 2004).

Studies conducted with *npr1-1* plants overexpressing an NPR1:green fluorescent protein (GFP) fusion suggest that NPR1-mediated *PR-1* activation requires the nuclear localization of the factor (Kinkema et al., 2000). Subsequent investigations established that under resting conditions the NPR1:GFP was confined to high molecular weight oligomeric complexes and found exclusively in the cytosol (Mou et al., 2003; Tada et al., 2008). Treatment with the SA analogue, INA, resulted in the accumulation of NPR1:GFP monomers within the nucleus (Kinkema et al., 2000; Mou et al., 2003). Residues Cys-82, Cys-156 and Cys-216 are required for the oligomerization of the NPR1:GFP, suggesting that disulfide bridging is responsible for the oligomeric conformation (Mou et al., 2003; Tada et al., 2008). When NPR1:GFP fusions mutated at any of these cysteine positions were overexpressed in the npr1 mutant background, the plants demonstrated constitutive nuclear localization of the GFP fusion and spurious activation of *PR-1* (Mou et al.,2003; Tada et al., 2008). Based on these NPR1:GFP studies it has been proposed that in the absence of infection, NPR1 exists as part of an oligomeric complex sequestered in the cytosol, but in response to SA accumulation the NPR1 is reduced to a monomeric conformation and translocated to the nucleus where it effects transcriptional reprogramming (Mou et al., 2003; Tada et al., 2008). In stark contrast to what was observed with the NPR1:GFP lines, anti-NPR1 immunoblot analysis of cytosolic and nuclear protein preparations from wild type *Arabidopsis* indicate that the NPR1 protein is

present in both the cytosol and nucleus under resting conditions (Després et al., 2000). These conflicting data demonstrate the need for further examination of the localization of NPR1 under non-inducing conditions because such information has important implications for the mechanism by which SA triggers the activation of NPR1.

While there is some debate as to the location of NPR1 protein under resting conditions, all data indicate that the SA-dependent *PR*-gene activation proceeds through a nuclear localized NPR1 (Després et al., 2000; Kinkema et al., 2000; Mou et al.,2003; Tada et al., 2008). Interestingly, the NPR1 protein lacks a known DNA binding domain yet possesses two protein-protein interaction motifs in the form of the N-terminal BTB/POZ domain and the central ankyrin repeats (Cao et al., 1997; Aravind and Koonin, 1999). Point mutations in either of the NPR1 protein-protein interaction motifs can render plants unable to induce *PR-1* or SAR (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997). These observations demonstrate the functional significance of protein interaction for the role of NPR1 in defense gene activation. The absence of a DB domain also suggests that the nuclear localized NPR1 is likely to operate as a coactivator, recruited and exerting its effect at the *PR-1* through a transcription factor.

The TGA2, TGA5 and TGA6 clade of basic leucine zipper (bZIP) transcription factors, named for their cognate DNA motif TGACG, are required for the activation of *PR-1* gene expression and SAR (Zhang et al., 2003). Much like an *npr1* mutant, plants knocked out in all three of these functionally redundant factors are unable to induce the expression of *PR-1* nor mount an effective SAR, in response to INA treatment (Zhang et al., 2003). These data would tend to indicate that the factors function as transcriptional activators of *PR-1* expression. However, under non-inducing conditions, the tga2/5/6

triple knock-out mutant plants demonstrate greater *PR-1* expression than that of wildtype plants, suggesting a role in transcriptional repression (Zhang et al., 2003). Importantly, the conflicting functions of the TGA2-clade are not manifested under the same conditions. Thus the data derived from the tga2/5/6 plant argue that the TGA2-clade could serve as dual function transcription factors.

Several TGA transcription factors, including the TGA2, TGA5 and TGA6, have been shown to interact with NPR1 (Zhang et al., 1999; Després et al., 2000; Zhou et al., 2000). NPR1 functional dissection experiments conducted with the yeast two-hybrid assay established that the ankyrin repeats domain was both necessary and sufficient for interaction with the TGA factors (Zhang et al., 1999). In contrast, the NPR1 BTB/POZ domain could not autonomously mediate TGA interaction, nor was it required for NPR1-TGA interaction (Zhang et al., 1999). In vitro pull down assays conducted with heterologously expressed factors demonstrated that TGA2 and NPR1 directly interact without the need of any bridging or adaptor proteins (Després et al., 2000). Using the fluorescence complementation assay (FCA), it was confirmed that NPR1 and TGA2 interact *in planta,* and importantly this system showed that the interaction takes place in the nucleus in response to SA treatment (Subramaniam et al., 2001). Notably, in all of the protein-protein interaction assays employed, it was found that those proteins encoded by the characterized *npr1* mutants were unable to mediate TGA interaction (Zhang et al., 1999; Després et al., 2000; Subramaniam et al., 2001) The fact that *npr1* mutants are unable to activate *PR-1* and SAR, and are also impaired for TGA interaction, strongly indicate that NPR1-TGA interaction is required for NPR1 function.

Functional dissection and linker-scanning (LS) mutagenesis have identified both positive and negative cis-acting elements, containing the TGA cognate binding sequence, in the *PR-1* promoter (Lebel et al., 1998). TGA binding sequences were found to occur in the LS5 and LS7 elements (Lebel et al., 1998). The former negatively regulates gene expression under both resting and activating conditions, and the latter is required for the INA-dependent gene induction (Lebel et al., 1998). The presence of negative and positive regulatory elements bearing the TGA cognate binding motif in the *PR-1* promoter provides some support for the dual function of the TGA2-clade of transcription factors in *PR-1* regulation. There is also evidence that the chromatin architecture at the promoter influences *PR-1* expression. Genetic approaches have established *SNI1* as a negative regulator of *PR-1* (Li et al., 1999). The *sni1* mutant plants were found to have elevated levels of the permissive chromatin marks H3 acetylation and H3K4 methylation at the *PR-1* promoter (Mosher et al., 2006). These chromatin modifications were credited for the elevated levels of *PR-1* expression reported in the *sni1* plants (Li et al., 1999; Mosher et al., 2006). Collectively these data demonstrate that promoter structures, both DNA and chromatin, contribute to *PR-1* expression.

Electrophoretic mobility shift assay (EMSA) experiments, performed with heterologously expressed proteins or those derived from the plant, demonstrated that NPR1 interaction with TGA factors stimulates the factors' binding affinity for DNA probes bearing the TGA cognate sequence (Després et al., 2000; Fan and Dong, 2002). Notably, the TGAs did not exhibit the binding enhancement when these experiments were conducted with proteins encoding *npr1* mutants (Després et al., 2000; Fan and Dong, 2002). *In planta* studies conducted with a chimeric TGA2 factor tethered to a

GAL4 DB found that this factor could stimulate reporter gene activation in an SA- and NPR1-dependent manner (Fan and Dong, 2002). Additionally, ChIP investigations showed that in the plant TGA2 could only be recruited to the *PR-1* in response to SA treatment, and this recruitment, like the binding enhancement and reporter gene activation, was contingent upon the presence of a functional NPR1 (Johnson et al., 2003). These findings suggest that the TGA2-clade of transcription factors serves as a surrogate DB domain for the NPR1, directing the master regulator to the *PR-1*. It is tempting to speculate that the TGA2-clade would function to fulfill the role of transcription activators enabling NPR1 to serve as a coactivator at the *PR-1*. However there is no evidence that the NPR1 is localized or recruited to the *PR-1* gene, nor has it been shown that the NPR1-TGA2 can assemble into a ternary complex in a relevant DNA context, such as the *PR-1*, *in planta*. Furthermore, by definition, coactivators function to activate gene expression by recruiting and stabilizing the transcription machinery or alteration of chromatin architectures (Roeder, 2005) and the present data have only demonstrated that the NPR1 enhances the DNA binding of TGAs. The biological significance and function of the NPR1 in the NPR1-TGA context remains unclear and largely unaddressed. The current description of TGA behaviour also fails to answer the question of how the TGA2-clade mediates the basal repression of *PR-1*. If the factor is not present at the *PR-1* under non-inducing conditions how can it effect the repression of this locus? A coactivator squelching-type repression mechanism also seems unlikely because the factor does not interact with NPR1, the master positive regulator of *PR-1* expression, under resting conditions (Subramaniam et al., 2001).

2.5 Expression of the *PR-10a* is the molecular marker for activation of the inducible defense response in potato

The *PR-10a* gene is activated in response to wounding, infection with the oomycete pathogen, *Phytophthora infestans*, or treatment with the pathogen derived elicitor arachidonic acid (AA) (Matton et al., 1993). The repression and activation of *PR-10a* expression are governed by different transcription factors operating through distinct cis-regulatory elements in the promoter. Repression of the *PR-10a* is mediated by the collective efforts of the SEBF and the cis-acting silencer element (SE) that spans nucleotides –52 and –27 of the promoter (Després et al., 1995; Boyle and Brisson, 2001). It is presumed that the SEBF functions to repress *PR-10a* expression through the SE element because the SEBF binds the SE element in vitro (Boyle and Brisson, 2001). However the recruitment of the SEBF to the SE promoter element has not been demonstrated in vivo.

The Why1 (StWhy1) transcription activator induces the expression of the *PR-10a* through the elicitor response element (ERE), which is located between nucleotides – 135 and – 105 (Desveaux et al., 2000; Desveaux et al., 2004). Under non-inducing conditions the Why1 is localized in the nucleus (Desveaux et al., 2000). However ChIP experiments have shown that under such conditions the nuclear localized Why1 is not associated with the *PR-10a* promoter (Desveaux et al., 2004). Prior to elicitation, the Why1 is likely to be distal from the *PR-10a,* sequestered in an inactive state by way of an uncharacterized inhibitor entity (Desveaux et al., 2000). The ChIP studies also demonstrated that the Why1 is only recruited to the promoter following wounding or elicitor treatment (Desveaux et al., 2004). It is unclear if the *PR-10a* promoter can simultaneously

accommodate the SEBF and Why1, or if the recruitment of these antagonistic entities is mutually exclusive. Such information would provide useful insights for the mechanism of *PR-10a* regulation.

Interestingly in the context of the EMSA, both the SEBF and Why1 behaved as strictly single stranded DNA binding proteins (Desveaux et al., 2000; Boyle and Brisson, 2001). The fact that both of the characterized transcriptional regulators of *PR-10a* are single stranded binding factors would tend to indicate that the *PR-10a* promoter exists in a single stranded or melted state. This uncommon conformation is likely to require unique regulatory mechanisms and presents a particularly interesting doorway for the RNAPII, which merits investigation.

2.6 Pto interacting protein 4 (Pti4) is an archetypical transcriptional activator of the inducible defense response in plants

The Pti4 was identified as a SEBF interactor by virtue of a yeast two hybrid screen (Brisson unpublished data) .The Pti4 factor was initially identified in a screen for interactors of the Pto kinase, which is responsible for conferring resistance to the bacterial speck disease in *Solanum lycopersicum* (tomato) (Zhou et al., 1997; Wu et al., 2002; Gu et al., 2002). Pti4 is a member of the ethylene response factor (ERF) family of transcription factors, which are found in a variety of plants species (Zhou et al., 1997; Gu et al., 2002). ERF factors are implicated in plant defense programs because they are induced in response to both biotic and abiotic stresses (Gu et al., 2002). A defining feature of these factors is the ERF DB domain. This domain specifically binds the GCC-

box cis element, which is a commonly occurring DNA motif in the regulatory region of many *PR* genes (Wu et al., 2002; Gu et al., 2002).

Ectopic expression studies performed with the tomato Pti4 in the model system *Arabidopsis* demonstrated that this factor activates the expression of an array of *PR* genes, the majority of which contain the GCC-box promoter motif (Wu et al., 2002; Gu et al., 2002). These results indicate that the Pti4 is a conserved transcriptional activator in plants. Intriguingly, ChIP experiments have shown that Pti4 recruitment is not limited to GCC-box containing promoters. One possible explanation for the Pti4's promoter promiscuity is that the factor is able to bind additional DNA motifs. However it could also be that the Pti4 is recruited to loci lacking the GCC-box motif through interactions with other transcription factors.

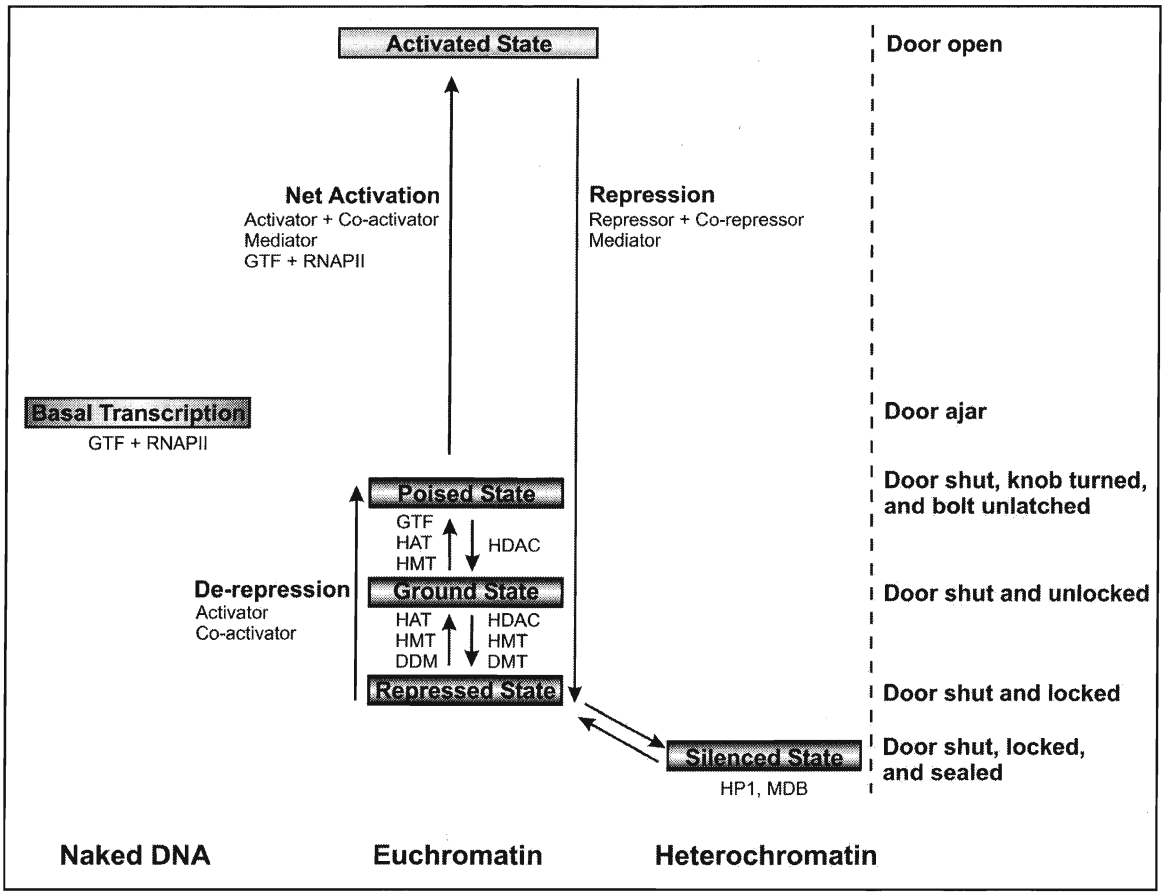

Figure 1. The door analogy for the states of gene activation.

The basal transcriptional machinery consists of the general transcription factors (GTF) and the RNA polymerase II (RNAPII). A naked DNA template in the presence of the basal transcriptional machinery demonstrates an intrinsic level of activation known as basal transcription. This state of gene activation is represented by a door ajar. In eukaryotes the door is effectively maintained in a closed position by way of chromatin structures, which prevent gene activation through the occlusion of the basal transcription machinery. Chromatin demonstrates what is referred to as the ground state of gene activation. Just as a shut door can be locked, the chromatin barrier can be further fortified through actions of repressors that enable the recruitment of corepressors demonstrating Histone Deacetylases (HDAC) Histone Methyltransferase (HMT) and DNA Methyltransferase (DMT) activities. The chromatin modifications mediated by these corepressors render the promoter in a repressed state. In addition to the occlusion of the basal transcription machinery and transcriptional activators, these chromatin modifications can also serve to recruit additional repressive entities including heterochromatin protein 1 (HP1) and methylated DNA binding proteins (MDB). The presence of these entities renders chromatin in a highly compacted and transcriptionally silent state known as heterochromatin. In this state the door is sealed shut. Gene activation, much like opening a locked door, is a multistep process. Unlocking the door requires clearance of repressors and the repressive chromatin modifications from the

promoter. Activators serve to recruit coactivators that boast histone acetyltransferase (HAT) and HMT and DNA demethylase (DDM) activities, which collectively contribute to the establishment of an open chromatin conformation permissive to the transcriptional machinery. Elements of the basal transcriptional machinery can also be recruited prior to RNAPII, creating a gene poised for activation. A fully activated state of gene expression is reached in response to the recruitment of the Mediator and the complete compliment of the basal transcriptional machinery, most notably the RNAPII, to the promoter. Adapted from Roeder (2005).

CHAPTER 3 – The Coactivator Function of Arabidopsis NPR1 Requires the Core of Its BTB/POZ Domain and the Oxidation of C-Terminal Cysteines

Contributions

This manuscript was born directly from my primary research project. I conducted the experiments and analysis that yielded Figure 1: A-F and I, Figure 2: A, C and D. I was involved in writing the results and discussion sections pertaining to Figures 1 and 2 and participated in the editing of the manuscript.

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3.1 ABSTRACT

NPR1 is the regulator of systemic acquired resistance (SAR) in *Arabidopsis*. Current models propose that following treatment with the SAR-inducing metabolite salicylic acid (SA), C82 and C216 of NPR1 are reduced, leading to nuclear import. Through an unknown mechanism, interaction of nuclear-localized NPR1 with TGA transcription factors results in the activation of defense genes, including the SAR marker *PATHOGENESIS-RELATED-1* (*PR-1*), and deployment of SAR. Aside from the fact that they interact with each other, there is no biochemical evidence indicating how TGA factors or NPR1 regulate transcription or whether a TGA-NPR1 complex forms on DNA. Here, we show by chromatin immunoprecipitation that TGA2 and NPR1 are recruited to the *PR-1* gene independently of each other and of SA-treatment. In vivo plant transcription assays revealed that TGA2 is not an autonomous transcription activator and that NPR1 de-represses *PR-1* expression in the absence of TGA2. TGA2 precludes the NPR1-dependent de-repression of *PR-1* in the absence of SA-treatment. However, after stimulation with SA, TGA2 is incorporated into a transactivating complex with NPR1 forming an enhanceosome. Genetic and biochemical data demonstrate that transactivation of the TGA2-NPR1 enhanceosome requires the core of the NPR1 BTB/POZ domain (residues 80-91) and the oxidation of NPR1 C521 and C529. These cysteines are found in a new type of transactivation domain that we term cysteine-oxidized transactivation domain. The data presented further our understanding of the mechanism by which TGA2 and NPR1 activate a disease-resistance gene.

3.2 INTRODUCTION

Plants, unlike animals, do not possess specialized cells for protection against invading pathogens. Instead, every plant cell must be capable of perceiving pathogens and mounting effective defense responses if the organism is to successfully protect itself from infection. Upon detection of an invading microbe, plant defense responses arise from the activation of signal transduction pathways that lead to global transcriptional reprogramming (Dangl and Jones 2001; Durrant and Dong 2004). Among the induced genes figure pathogenesis-related (*PR*) genes which are activated both at the site of infection and in uninfected parts of the plant in response to the pathogen-induced accumulation of salicylic acid (SA) (Ryals et al., 1996). Local and distal SA accumulations are mandatory to the deployment of a systemic long-lasting and broad-spectrum plant disease resistance response called systemic acquired resistance (SAR) (Durrant and Dong 2004; Pieterse and Van Loon 2004; Ryals et al., 1996). Exogenous application of SA, termed chemical SAR, also triggers *PR* gene induction and SAR deployment (Ward et al., 1991).

The NPR1 protein is the key regulator of SAR (Cao et al., 1994; Delaney et al., 1995). In resting cells of wild-type *Arabidopsis*, NPR1 is found in both the cytoplasm and the nucleus (Després et al., 2000). However, in an *npr1-1* mutant line of *Arabidopsis* overexpressing an NPR1–GFP fusion protein, the NPR1 fusion is sequestered in the cytoplasm and only localizes to the nucleus after SA treatment (Kinkema et al., 2000). The cytoplasmic NPR1–GFP fusion protein is contained within an oligomer complex held together by disulfide bridges (Mou et al., 2003). Upon SA treatment, NPR1 C82 and

C216 are presumably reduced and NPR1-GFP is released from this complex, resulting in accumulation of protein monomers inside the nucleus (Mou et al., 2003).

Activation of *PR* genes during SAR, which requires the nuclear localization of NPR1 (Kinkema et al., 2000), is also dependent on a functionally redundant clade of three basic leucine zipper TGA transcription factors, TGA2, TGA5, and TGA6, that interact with NPR1 (Després et al., 2000; Zhang et al., 1999). A triple knock-out of these TGA genes abolished *PR-1* induction by SA, indicating that the gene products could act as transcriptional activators (Zhang et al., 2003). This conclusion is supported by a report in which a chimeric TGA2-GAL4:DB protein was used to study gene regulation and proposed to act as a transcriptional activator (Fan and Dong 2002). However, in a finding that appears to be contradictory to the previous one, Zhang et al., (2003) showed that whether unstimulated or SA-treated, the triply knocked-out plants displayed higher levels of *PR-1* (when compared to levels found in wild-type without SA), which could indicate that the proteins of the TGA2-containing clade act as repressors of *PR-1*, presumably by binding to its promoter (Zhang et al., 2003). Furthermore, chromatin immunoprecipitation (ChIP) experiments have demonstrated that TGA2 physically interacts with the *PR-1* promoter in an SA- and NPR1- dependent manner (Johnson et al., 2003), which would also contradict the hypothesis that TGA2 binds to the *PR-1* promoter in the absence of SA (Zhang et al., 2003). It is thus not clear whether TGA2 is a transcriptional activator or a repressor. *PR-1* is also positively regulated in an SA-dependent, but NPR1-independent fashion by the transcription factor AtWhy1 (Desveaux et al., 2004). Furthermore, *PR-1* is negatively regulated by SUPPRESSOR OF NPR1 INDUCIBLE1 (SNI1; Li et al., 1999) and ChIP experiments have shown an increase in

histone H3 acetylation and methylation at the *PR-1* promoter in *sni1* mutant plants (Mosher et al., 2006). These data implicate chromatin structure in the regulation of *PR-1* expression.

NPR1 and TGA factors (TGA1 and TGA2) physically interact within the nucleus and in vitro (Després et al., 2003; Fan and Dong 2002; Subramaniam et al., 2001). This interaction stimulates the DNA-binding activity of TGA factors to their cognate cis-acting element in vitro (Després et al., 2000; Després et al., 2003) and in vivo (Fan and Dong 2002). However, because NPR1 does not form a stable complex with TGA2 and its cognate DNA in electrophoretic mobility shift assays (Després et al., 2000), it is unclear whether, when inside the nucleus, NPR1 and TGA2 interact only in the nucleoplasm or whether they can form a ternary complex on the DNA (DNA-TGA2-NPR1 complex). There is also no experimental evidence indicating that NPR1 is actually recruited to the *PR-1* gene in vivo. This has led to the proposition that NPR1 could act as a crowbar chaperone facilitating the binding of TGA factors to DNA and implying that NPR1 would not form a stable ternary complex with a TGA factor bound to DNA (Sehnke et al., 2005). Therefore, aside from its DNA-binding enhancement activity on TGA factors, the biochemical role of NPR1 in NPR1-TGA complexes, if any, remains speculative.

NPR1 contains two protein-protein interaction motifs: ankyrin repeats (Cao et al., 1997; Mosavi et al., 2004; Ryals et al., 1997) and a BTB/POZ (Broad-Complex, Tramtrack, and Bric-a-brac/Pox virus and Zinc finger) domain (Aravind and Koonin 1999; Bardwell and Treisman 1994). The ankyrin repeats mediate interactions with TGA factors and their mutation abolishes NPR1-TGA complex formation, *PR* gene expression, and SAR (Cao et al., 1997; Després et al., 2000; Després et al., 2003; Ryals et al., 1997;

Zhang et al., 1999). The functional requirements of the NPR1 BTB/POZ in disease resistance are not yet understood.

Here, we demonstrate that TGA2 is not a transcriptional activator in resting or SA-treated cells, as it is unable to activate transcription when expressed on its own. We show that TGA2 and NPR1 can, independently of one another, physically interact with the *PR-1* promoter in both resting and SA-treated cells. We also show that NPR1 contains an autonomous transactivation domain in its C-terminus and acts as a co-activator in SA-treated cells where it associates with TGA2 to create a transcriptional activating complex. NPR1 and TGA2 are sufficient to activate gene expression after stimulation of the cells with SA and thus the DNA-TGA2-NPR1 ternary complex constitutes an SA-dependent enhanceosome. We demonstrate that the co-activator function of NPR1 requires the presence of the BTB/POZ core and the oxidation of C521 and C529, located in the transactivation domain of NPR1. Finally, using an in vivo labeling technique capable of distinguishing between the reduced and oxidized state of cysteines, we determined that C521 and C529 are oxidized in both resting and SA-treated cells. The data presented here not only provide a mechanistic understanding of transcriptional regulation mediated by the TGA2-NPR1 complex but also help to elucidate the biochemical function of TGA2, a repressor of NPR1-mediated de-repression, NPR1, a co-activator, and to unravel the existence of a new type of eukaryotic transactivation domain that we term: cysteine-oxidized transactivation domain.

3.3 RESULTS

3.3.1 Recruitment of TGA2 to the PR-1 Promoter is Both SA- and NPR1-Independent

SA-induction of the *PR-1* gene is positively controlled by a clade of three TGA factors (TGA2, 5 and 6) with redundant functions. In the triple TGA knock-out plants, the levels of *PR-1* transcripts were up to 50-fold higher, when compared to non-stimulated wild-type plants (Zhang et al., 2003). This was interpreted as a loss of TGA factor binding to a negative element in the *PR-1* promoter; however, whether this effect was due to direct binding of the TGA factors to DNA was not addressed. If the interpretation of Zhang et al. (2003) is correct, their results would contradict those of Johnson et al. (2003), who demonstrated using ChIPs that recruitment of TGA2 to the *PR-1* promoter is both SA- and NPR1-dependent. These ChIPs were performed on endogenous TGA2 using an anti-TGA2 antibody raised against the N-terminus. However, since ChIPs can generate false negatives when epitopes are inaccessible, we sought to determine whether the apparent lack of interaction between TGA2 and the *PR-1* promoter in resting cells observed by Johnson et al. (2003) is due to the absence of antibody recognition, to masking of the epitope, or to the absence of TGA2.

As a means of generating an alternative epitope, the *TGA2* coding region was ligated to the one encoding the Gal4 DNA-binding domain (DB) and the resulting fusion (TGA2:DB), under the control of the *CaMV35S* promoter, was introduced into the *tga2/5/6* knock-out plants. Figure 1A is a diagram of the *PR-1* gene which shows the position of the PCR primers used for all the ChIP experiments. Figure 1B shows that a PCR product is present in the lanes corresponding to immunoprecipitations performed with the anti-Gal4 antibody (lanes 3 and 7), indicating that TGA2:DB interacted with *PR-1* in both untreated and SA-treated cells. Immunoprecipitation with pre-immune serum

(PI) did not lead to a detectable band (lanes 2 and 6). ChIP performed with the anti-Gal4 antibody on the untransformed *tga2/5/6* mutant plant also did not lead to a detectable band (data not shown).

The above results indicate that the lack of interaction previously reported by Johnson et al. (2003) in the absence of SA was due to the masking, under certain conditions, of the N-terminal TGA2 epitope chosen by these authors. Therefore, it became relevant to test whether the same phenomenon was responsible for the lack of interaction reported between TGA2 and *PR-1* in the *npr1* background (Johnson et al., 2003). To do so, TGA2:DB, under the control of the *CaMV35S* promoter, was introduced in the *npr1-3* mutant background and ChIPs were performed using the anti-Gal4 antibody. Results of Figure 1C show the presence of a PCR product in the lanes corresponding to immunoprecipitations performed with the anti-Gal4 antibody (lanes 3 and 7). This indicates that, in the absence of NPR1, TGA2:DB interacted with *PR-1* in both untreated and SA-treated cells. Immunoprecipitation with PI did not lead to a detectable band (lanes 2 and 6). ChIP performed with the anti-Gal4 antibody on the untransformed *npr1-3* mutant plant did not lead to a detectable band (data not shown).

3.3.2 Recruitment of NPR1 to the PR-1 Promoter is both SA- and TGA2/5/6-Independent

Since no experimental evidence exists to indicate that NPR1 can be recruited to the *PR-1* promoter, it is unclear whether NPR1 is capable of forming a complex with TGA2 on DNA to modulate transcription. To address this question, we performed ChIP

experiments with wild-type and *npr1-3* mutant *Arabidopsis* plants, before and after SA-treatment (Figure 1D). The *npr1-3* mutant was chosen as a negative control since this allele carries a premature stop codon (Cao et al., 1997), which removes the amino acid region used to raise the anti-NPR1 antibody (Després et al., 2000). The specificity of the anti-NPR1 antibody has been demonstrated previously (Després et al., 2000) and can also be witnessed in Figure 2C, where a band corresponding to NPR1 was detected in the wild-type plant (lane 1) but not in the *npr1-3* mutant plant (lane 2). With the exception of input lanes (lanes 1 and 7), ChIP performed on the *npr1-3* lines did not yield a band, regardless of whether cells were treated with SA or whether the immunoprecipitation antibodies were from PI or raised against NPR1. Conversely, ChIP performed on wild-type plants indicated that NPR1 interacted with *PR-1* in both untreated cells and cells treated with SA (lanes 6 and 12). Immunoprecipitation with PI did not lead to a detectable band (lanes 5 and 11).

Intuitively, knowing that NPR1 and TGA2 can interact with each other and because NPR1 does not contain a known DNA-binding domain, one could expect the recruitment of NPR1 to the *PR-1* promoter to be dependent on TGA2. To test this hypothesis, we performed ChIP experiments on the *tga2/5/6* mutant plant using the anti-NPR1 antibody. The presence of a PCR product in the lanes corresponding to immunoprecipitations performed with the anti-NPR1 antibody (Figure 1E, lanes 3 and 7) indicate that NPR1 continues to interact with *PR-1* in the absence of TGA2/5/6, in both untreated and SA-treated cells. Immunoprecipitation with PI did not lead to a detectable band (lanes 2 and 6). Note that formaldehyde, the cross-linker used in the ChIP experiments, can cross-link protein to DNA but also protein to protein (Buck and Lieb,

2004). Hence, recruitment of NPR1 to the *PR-1* promoter does not indicate that NPR1 binds directly to DNA.

3.3.3 NPR1 Is a Co-Activator Required for Transcriptional Activation by a TGA2-NPR1 Complex in SA-Treated Cells Only

PR-1 is positively regulated by NPR1 (Cao et al., 1997; Ryals et al., 1997) and by TGA2/5/6 (Zhang et al. 2003). This prompted us to test whether TGA2 can act as a transcriptional activator. To do so, TGA2:DB was assayed using an in vivo plant transcription assay (Figure 1F). The baseline level of transcription was determined by transfecting leaves with Gal4 DB (not fused to any other protein or protein domain) along with a reporter construct consisting of a firefly luciferase gene under the control of 5 copies of the Gal4 upstream activating sequences (UAS) fused to a minimal promoter. Transfection with TGA2:DB did not result in reporter gene activation beyond the baseline level, regardless of whether cells were treated with SA or not. The same result was obtained with TGA2 that was not fused to Gal4 DB or any other foreign protein domain (TGA2). Transfection with Gal4 DB fused to a strong transactivation domain (Gal4 DB:VP16 TA) led to SA-independent expression of the reporter gene well above the baseline (Figure 1F, white versus grey bars). These results demonstrate that the reporter gene can indeed be activated under our experimental conditions and indicate that TGA2 is not a transcriptional activator, whether or not cells are stimulated with SA.

Knowing that NPR1 can be recruited to a promoter in vivo (Figure 1D and E), we tested if NPR1 can activate transcription when tethered to DNA. To accomplish this,

NPR1 was fused to Gal4 DB (NPR1:DB) and assayed using the in vivo plant transcription assay (Figure 1G). In untreated cells (white bars), NPR1:DB did not lead to gene activation beyond the baseline level. However, after SA treatment (grey bars), NPR1:DB activated transcription 2.2-fold above the baseline level. Expression of NPR1 without fusion to Gal4 DB (NPR1) did not lead to gene activation that was significantly different from the baseline level ($p<0.05$), indicating that transactivation by NPR1:DB observed with SA was dependent on the recruitment of NPR1 to the promoter. The results indicate that NPR1 could potentially act as a transcriptional co-activator if recruited to a promoter via a DNA-binding protein, such as TGA2.

We next addressed whether NPR1 could modulate the transcriptional properties of TGA2. When TGA2:DB was co-expressed with NPR1 (not fused to any foreign transcription activation or DNA-binding domains), expression of the reporter gene in untreated cells did not increase beyond the baseline (Figure 1H, white bars). However, transcription rose 2.6-fold above the baseline level after SA treatment (Figure 1H, grey bars). Since neither TGA2:DB nor NPR1 activate transcription of the reporter gene on their own (Figure 1F and 1G) and NPR1 stimulates transcription when tethered to DNA (Figure 1G, NPR1:DB), results from Figure 1H suggest that the transcriptional activation observed when NPR1 (unfused) is co-expressed with TGA2:DB is likely due to NPR1 being tethered, or recruited, to the DNA through TGA2:DB. Physical interaction between TGA2 and NPR1 at the reporter gene promoter and in the presence of SA was demonstrated using plant two-hybrid assays (Figure 1I, TGA2:DB + NPR1:TA). Together, these observations are consistent with the formation of a ternary complex

between DNA, TGA2:DB and NPR1, with NPR1 acting as a co-activator of TGA2 on the Gal4-based promoter.

Using plant two-hybrid assays (Figure 1I), we showed that, in the absence of SA (white bars), NPR1 fused to VP16 TA (NPR1:TA) also interacted with TGA2:DB (significant difference $p<0.05$ between TGA2:DB and TGA2:DB + NPR1:TA), but very poorly. A similar conclusion was reached based on data from a protein fragment complementation assay (Subramaniam et al., 2001). Thus, in addition to the fact that NPR1:DB (tethered to DNA) does not transactivate in the absence of SA, the very weak interaction between NPR1:TA and TGA2:DB in unstimulated cells may also account for the lack of transcriptional stimulation by NPR1. We also confirmed that, in the absence of SA, NPR1:TA is competent to interact with other proteins as demonstrated by its interaction with a mutant version of TGA1 (Figure 1I, TGA1m:DB), which was previously shown to interact with NPR1 in the presence and absence of SA-treatment (Després et al., 2003).

Next, we tested the transactivation properties of NPR1 and TGA2 in the context of the *PR-1* promoter. DNA coding for native (unfused) proteins were delivered by biolistics as in Figure 1F-1I, except that the reporter consisted of the luciferase gene under the control of the *PR-1* promoter (Figure 1J). Relatively low levels of luciferase activity were detected following transfection of this reporter gene without effector plasmids (-). Transfection of an unrelated effector, Gal4 DB, which does not bind *PR-1* (there is no Gal4 binding site in the *PR-1* promoter), increased reporter gene expression, which most likely represents the unspecific effect of expressing a protein in this system. Thus, Gal4 DB was used as the baseline for this system. Whether cells were treated (grey

bars) or not (white bars) with SA, NPR1 led to activation of the *PR-1* promoter beyond the baseline level. TGA2, on the other hand, had no effect on the baseline activity of the promoter. However, in untreated cells (white bars), when NPR1 was co-expressed with TGA2, transcription values were brought back down to the baseline level, indicating that TGA2 repressed the NPR1-dependent activation of *PR-1*. As observed with NPR1, protein nim1-2, a variant of NPR1 with a mutation in an ankyrin repeat and which does not interact with TGA2, also activated the *PR-1* promoter in the absence of TGA2 in untreated and SA-treated cells. This suggests that the ankyrin repeats are unlikely to be involved in the recruitment of NPR1 to the *PR-1* promoter. Furthermore, since nim1-2 does not interact with TGA2, this result is also consistent with a TGA2-independent recruitment of NPR1 to the *PR-1* promoter, as was observed with ChIPs (Figure 1E). Co-expression of nim1-2 with TGA2 also restored transcription values to the baseline level. Co-expression of TGA2 and NPR1 in SA-treated tissues (grey bars) led to activation of *PR-1* beyond the baseline and significantly beyond what was observed with NPR1 alone ($p<0.05$), confirming that NPR1 acts as a TGA2-co-activator on the *PR-1* promoter. Also, co-expression of TGA2 with NPR1:TA established that the two proteins interact on the *PR-1* promoter, only in the presence of SA (grey bars), since values observed with TGA2 + NPR1:TA were significantly higher than those obtained with TGA2 + NPR1 ($p<0.05$) or NPR1:TA alone ($p<0.05$). Our results indicate that, in untreated cells, TGA2 represses the NPR1-dependent activation of *PR-1*, without the two proteins interacting with each other. However, after SA-treatment, the two proteins interact to form a ternary complex, with *PR-1* DNA, in which NPR1 acts as a TGA2-co-activator.

3.3.4 The BTB/POZ Domain of NPR1 Is Required for PR-1 Activation by SA

To determine the functional importance of the NPR1 BTB/POZ domain, we generated a series of rational mutants based on information available from other model systems. Of the four known structural classes of BTB domains (BTB Zinc Finger, Skp1, ElonginC, and T1), NPR1 is more similar to those associated with zinc fingers, the so-called long-form (Stogios et al., 2005). We thus performed a small scale multiple alignment (Figure 2A) of long-form BTB/POZ domains including the one from human promyelocytic leukemia zinc finger (PLZF), the archetypical BTB/POZ domain (see Aravind and Koonin, 1999 for a more exhaustive alignment of 79 BTB/POZ domains including that of NPR1). Also shown is a representation of the secondary structure of the PLZF BTB/POZ derived from its crystal structure (Ahmad et al., 1998; PDB accession code: 1buo).

Since the N-terminal region of the NPR1 BTB/POZ is longer than that of PLZF, we used the protein secondary structure prediction PSIPRED (Jones, 1999) and identified a potential β-strand formed by residues 19-22 (FVAT). Deletion of this putative structure generated the Δ22 mutant (Figure 2A). The next deletion, corresponding to the Δ44 mutant, removed β1, which has been shown to partially destabilize the PLZF dimer (Ahmad et al., 1998). Deletion mutant Δ66 removed all the structural determinants (β1, α1, and D65) mandatory for BTB/POZ homodimerization (Ahmad et al., 1998). The α2 and α3 helices are buried within the BTB/POZ and constitute the monomer core of the domain (Ahmad et al., 1998). Alanine-substitution of the core in PLZF results in disruption of the BTB/POZ fold (Melnick et al., 2000). The core region is well conserved in NPR1, and of note, the sequence "RSSFF", residues 87-91 of NPR1, is identical to the

corresponding region in POZ3, and the sequence "HRCVL" residues 80-84, identical to the corresponding region in ZF5. Thus, to permit functional testing of the NPR1 BTB/POZ core without deleting other elements, the conserved residues in α2 and α3 were substituted to alanines (Figure 2A, Alanine-Substitution brackets). Finally, since β2, β3, and β4 form a tertiary structure, an N-terminal deletion aimed at removing the core of the BTB/POZ was created after β4 but before the next structural element (Δ110 deletion mutant). The five NPR1 variants mutated in the BTB/POZ (Δ22, Δ44, Δ66, Δ110, and the alanine-substitution), all interacted with TGA2 in yeast two-hybrid assays (Figure 2A inset). Quantitative yeast two-hybrid tests confirmed that the five NPR1 mutants interacted with TGA2. However, the data also indicated that these mutants did not interact with TGA2 as well as did the full-length wild-type NPR1 (Supplemental Figure 1).

In order to assess the biological significance of the NPR1 BTB/POZ in controlling *PR-1* expression, we created and tested five cDNA constructs encoding the proteins depicted in Figure 2A. These were introduced, under the control of the *CaMV35S* promoter, into the *npr1-3* genetic background (Figure 2B). As a control, *npr1-3* plants were transformed with the full-length, wild-type *NPR1* coding region fused to the *CaMV35S* promoter (NPR1, lanes 3 and 4). Wild-type *Arabidopsis* (WT) accumulated *PR-1* transcripts when treated with 0.5 mM SA for 16 hr (lane 1), while *npr1-3* plants (NPR1-3) did not (lane 2). *PR-1* gene expression was restored in 21 of the 25 independent transgenic *npr1-3* lines expressing NPR1 (lanes 3 and 4, and data not shown), in all 23 lines expressing Δ22 (lanes 13 and 14, and data not shown), in 35 of 38 lines expressing Δ44 (lanes 11 and 12, and data not shown), in 18 of 24 lines expressing

Δ66 (lanes 9 and 10 and, data not shown), but in none of the 31 and 40 independent lines expressing the alanine-substituted BTB/POZ (lanes 5 and 6, and data not shown) or Δ110 (lanes 7 and 8, and data not shown), respectively. Panels C and D indicate that the Δ110 and alanine-substitution proteins were expressed in these lines. *PR-1* transcripts were not detected in any of the lines tested in the absence of SA (data not shown).

Altogether, the results of Figure 2 indicate that although alanine-substitution and Δ110 can interact with TGA2 (Figure 2A, inset and Supplemental Figure 1), their expression cannot complement the *npr1-3* mutation, demonstrating that the interaction of NPR1 with TGA2 is in itself not sufficient for biological activity, and that the core of the NPR1 BTB/POZ, in the context of the full-length NPR1, is required for *PR-1* induction.

3.3.5 The NPR1 BTB/POZ Core Is Required for the TGA2-Co-Activator Function of NPR1 in SA-Treated Cells

To establish a link between the complementation of *PR-1* expression and the transactivation of the TGA2-NPR1 complex, we determined whether the deletions and the alanine-substitution of the NPR1 BTB/POZ affected the capacity of this protein to act as a TGA2-co-activator. Deletions of the first 22, 44, or 66 amino acids of NPR1 did not substantially affect the capacity of NPR1 to convert TGA2:DB into an activator after treatment with SA (Figure 3A). However, deleting the first 110 amino acids or substituting the BTB/POZ core with alanines abolished transactivation of the co-expressed TGA2:DB (Figure 3A). The nim1-2 protein, which does not interact with TGA2, served as a negative control. In the absence of SA-treatment, none of the mutants

significantly altered transactivation of TGA2:DB compared to results obtained with full-length NPR1 (Figure 1F) and, accordingly, data are not shown. We also tested the alanine-substitution and Δ110 proteins for their capacity to interact with TGA2 in the plant two-hybrid system (Figure 3B), which evaluates interaction in the context of the promoter. The data indicate that alanine-substitution and Δ110 fused to VP16 TA interacted with TGA2:DB with no significant differences in the level of interaction ($p<0.05$) when compared to Δ22, Δ44 and Δ66. However, the interaction of these five mutant proteins with TGA2 was significantly lower from that of wild-type NPR1 ($p<0.05$). These results are consistent with those obtained with quantitative yeast two-hybrid assays (Supplemental Figure 1). Together, the findings shown in Figure 3 indicate that amino acids located between residues 66 and 110 of NPR1, more precisely residues 80 to 84 and/or 87 to 91, which constitute the core of the BTB/POZ, are required for the TGA2-co-activator function of NPR1.

3.3.6 NPR1 Harbors a Cryptic Transactivation Domain in its Last 80 Amino Acids

Given that the core of the NPR1 BTB/POZ is required for transactivation of the TGA2-NPR1 complex, we sought to determine whether this domain harbors autonomous transcriptional regulatory regions. To identify these potential regulatory regions, the NPR1 BTB/POZ (amino acids 1 to 190) was fused to Gal4 DB (POZ:DB) and assayed using the in vivo plant transcription assay (Figure 4A). In the absence (white bars) or presence (grey bars) of SA-treatment, POZ:DB and variants, in which the first 22, 66, and 110 amino acids were deleted (Δ22POZ:DB, Δ66POZ:DB, Δ110POZ:DB) or in which

the core of the BTB/POZ was replaced with alanines (A-SubPOZ:DB), did not stimulate transcription beyond the baseline level (Gal4:DB). One of the most salient features of this experiment was the uncovering of a cryptic transactivation domain, revealed when the BTB/POZ was shortened by 44 amino acids at the N-terminus (Δ44POZ:DB), suggesting that a repressing element is located between amino acids 22 and 44. However, in SA-treated cells, Δ44POZ:DB did not transactivate (Figure 4A; Δ44POZ:DB, grey versus white bars), indicating that the cryptic transactivation domain does not function when cells are induced with SA. Taken together, the results of Figure 4A indicate that the BTB/POZ domain cannot account for the transactivation properties of the full-length NPR1 tethered to DNA through the Gal4 DB.

Having determined that the BTB/POZ domain does not harbor an autonomous transactivation domain active in SA-stimulated cells (Figure 4A), we set out to identify such domains in the C-terminal portion of NPR1. We created additional N-terminal deletions of NPR1 (Figure 4B); one at amino acid 373, which occurs right after the ankyrin repeats as predicted by Pfam (Finn et al., 2006) and SMART (Letunic et al., 2006); one after residue 463, which is the end point of sequence similarity with Drosophila Ankyrin 2 (Genbank accession number: AAN12046.1); one at position 513, which corresponds to the beginning of the last stretch of negatively charged and hydrophobic residues, a signature of transactivation domain (Cress and Triezenberg 1991); and finally, one right before the nuclear localization signal (Kinkema et al., 2000), at amino acid 533. These constructs were fused to Gal4 DB and assayed using the in vivo plant transcription assay (Figure 4C). In unstimulated cells (Figure 4C, white bars), deletion of the first 373 or 463 amino acids of NPR1 (Δ373:DB and Δ463:DB), did not

show gene activation beyond the baseline level. However, further deletion to residue 513 (Δ513:DB), resulted in gene activation 2.2-fold above the baseline level, indicating that a repressing region had been deleted, thus exposing a cryptic transactivation domain. Extending the deletion to position 533 (Δ533:DB) reduced gene activity to the baseline level, emphasizing the importance of residues 513 to 533 for transactivation.

In SA-stimulated cells (Figure 4C, grey bars), deletion of the first 373, 463, or 513 amino acids of NPR1 (Δ373:DB, Δ463:DB, or Δ513:DB) resulted in gene activation 1.6-fold above the baseline level. Extending the deletion to position 533 (Δ533:DB), reduced gene activity to the baseline level, again indicating the importance of residues 513 to 533 for transactivation. The results of Figure 4C demonstrate that, in addition to amino acids 22-44 in the BTB/POZ, NPR1 possesses a second repression region, located between position 463 and 513, and active in unstimulated cells only, as these regions do not bring about repression in the SA-treated cells. Furthermore, a transactivation domain, active in uninduced as well as in SA-stimulated cell, requires residues located between position 513 and 533.

3.3.7 Oxidation of NPR1 Cysteines 521 and 529 Is Required for the Activity of the Transactivation Domain in SA-Treated Cells only

Inspection of the region containing the C-terminal transactivation domain of NPR1 reveals that it contains two cysteine residues (Figure 5A), at position 521 (C521) and 529 (C529). Since cysteines can be subjected to redox modifications that affect protein function, we first set out to determine whether C521 and C529 were required for the

transactivation of the last 80 amino acids of NPR1 tethered to DNA (Δ513:DB). C521 and C529 (the only two NPR1 cysteines found in Δ513:DB) were individually mutated to a serine, an amino acid similar to cysteine, in size and structure, but lacking the ability for redox modifications. Hence, serine can mimic the reduced form of cysteine and preserve the capability for hydrogen bonding.

The constructs bearing a mutated cysteine were fused to Gal4 DB and assayed using the in vivo plant transcription assay (Figure 5B). In resting cells (Figure 5B, white bars), mutation of cysteines at positions 521 (Δ513C521S:DB) or 529 (Δ513C529S:DB) had no effect on gene activation, with levels similar to Δ513:DB (no difference at $p=0.05$), indicating that redox modulation of C521 and C529 does not play a role in transactivation under non-induced conditions. However, in SA-treated tissues (Figure 5B, grey bars), Δ513C521S:DB and Δ513C529S:DB did not lead to gene activation beyond the baseline level and values were significantly different from Δ513:DB ($p<0.05$). These results indicate that, in the context of the last 80 amino acids of NPR1, C521 and C529 are required for transactivation only after SA-treatment. To establish the redox status of C521 and C529, we performed a labeling technique that distinguishes between protein sulfhydryls (reduced Cys residues) and disulfides (oxidized Cys residues) (see Després et al., 2003 for a flow chart and description of the method). The results (Figure 5C) indicate that cysteine residues in the last 80 amino acids of NPR1 are predominantly oxidized (Ox), whether or not the cells have been treated with SA.

We next tested the effect of their mutations in the context of the full-length NPR1 tethered to DNA by the Gal4 DB (Figure 5D). In unstimulated cells (white bars), NPR1:DB did not lead to transactivation beyond baseline levels whether or not the

cysteines were mutated. However, after SA-treatment, in contrast to what is observed with NPR1:DB, mutations of these cysteines abolished transactivation and values were significantly different from wild-type NPR1:DB ($p<0.05$). Plant two-hybrid experiments confirmed that C521S:DB and C529S:DB were expressed and retained the capacity to interact with TGA2 to an extent comparable to wild-type NPR1:DB (Figure 5E). These results suggest that the TGA2-co-activator function of NPR1 may require C521 and C529.

3.3.8 Transcriptional Activation of the PR-1 gene and TGA2-Co-Activator Function of NPR1 Require Cysteines 521 and 529 of NPR1

Finally, we sought to determine whether mutating C521 and C529 would affect the TGA2-co-activator function of NPR1. We first tested the role of these cysteines in the context of the *Gal4* promoter and observed that mutation of C521 or the double mutation C521/C529 abolished the capacity of the TGA2-NPR1 complex to transactivate (Figure 5F). Plant two-hybrid experiments confirmed that constructs C521S and C521S/C529S retained the capacity to interact with TGA2 to an extent comparable to wild-type NPR1 (Figure 5G) in the configuration where TGA2 is fused to the Gal4 DB. Next, we tested the role of these cysteines in the context of the *PR-1* promoter. DNA coding for native (unfused) proteins were delivered by biolistics along with the luciferase reporter gene under the control of the *PR-1* promoter (Figure 5H). As observed with NPR1, proteins C521S and C521S/C529S activated the *PR-1* promoter in the absence of TGA2 whether or not cells were treated with SA, indicating that these residues of NPR1 are not required

for recruitment to the promoter. Under induced (grey bars) and non-induced conditions (white bars), mutation of these cysteines did not bring about transactivation of the complex as values were not significantly different from those obtained with TGA2 alone (at $p=0.05$). The results observed after SA-treatment (grey bars) were significantly different ($p<0.05$) from those obtained with the TGA2-NPR1 complex, which activated the *PR-1* promoter, as values were significantly greater ($p<0.05$) than those observed with TGA2 alone or NPR1 alone.

To further confirm the biological significance of C521 and C529 of NPR1 in controlling *PR-1* expression, an NPR1 construct harboring the double mutation at cysteines 521 and 529 was introduced, under the control of the *CaMV35S* promoter, into the *npr1-3* genetic background (Figure 5I; C521/529m). Wild-type *Arabidopsis* (WT) expressed *PR-1* transcript when treated with 0.5 mM SA for 16 hr (lane 1), while *npr1-3* plants (NPR1-3) did not (lane 2). *PR-1* gene induction was not restored in any of the 19 independent transgenic *npr1-3* lines expressing C521/529m (lanes 3 and 4, and data not shown). Figure 5J indicates that protein C521/529m was expressed at levels similar to those observed with NPR1. None of the lines tested expressed *PR-1* in the absence of SA (data not shown).

3.4 DISCUSSION

Our study has demonstrated that TGA2 is not a transcriptional activator whether cells are resting or SA-treated. Furthermore, our data argue that, upon SA-treatment, *PR-1* is up-

regulated by a transactivation complex composed of at least TGA2 and NPR1. First, ChIP in wild-type *Arabidopsis* confirmed that NPR1 is recruited to the *PR-1* promoter in both non-treated and SA-treated cells. Second, despite the fact that TGA2 is not a transactivator, NPR1 associates with TGA2 in SA-stimulated cells to form a transcriptional activating complex, both on a heterologous (5X Gal4 UAS) and a native (*PR-1*) promoter. Third, genetic complementation analyses of rationally designed site-directed and deletion mutants of the NPR1 BTB/POZ established a role for the core of this domain in activating *PR-1*. This finding is important because it establishes a direct correlation between complementation of *PR-1* expression and transactivation on the heterologous promoter of a complex containing TGA2 and these NPR1 BTB/POZ mutants. Fourth, a cysteine-oxidized transactivation domain in the C-terminus of NPR1 is also required for the activation of *PR-1* by the TGA2-NPR1 complex. This emphasizes again the correlation between transactivation of the TGA2-NPR1 complex and the activation of *PR-1*. We thus conclude that, in SA-treated cells, NPR1 is a TGA2-co-activator essential for *PR-1* induction.

3.4.1 TGA2 Is Required for Transcriptional Repression of PR-1 in Uninduced Cells

The observation that, under uninduced and SA-induced conditions, the triply knocked-out *tga2/5/6* mutant displayed levels of *PR-1* expression 50-fold high than in the wild-type suggested that TGA2, and members of its clade, could act as transcriptional repressors (Zhang et al., 2003), which implied that they can bind the *PR-1* promoter independently of treatment with SA. Our ChIP results using TGA2 fused to a Gal4 DB epitope (Figure

1B and C) indeed indicate that recruitment of TGA2 to the *PR-1* promoter is both SA- and NPR1-independent and thus suggest that the de-repression of *PR-1* observed by Zhang et al. (2003) in the *tga2/5/6* knock-out plant is due to the lack of direct binding of these TGA factors to *PR-1*. However, this contradicts a report in which ChIP indicated that binding of TGA2 to *PR-1* is both NPR1- and SA-dependent (Johnson et al., 2003). Since the N-terminal region of TGA2 used by Johnson et al. (2003) to raise the anti-TGA2 antibody contains 28% of serine and threonine, two phosphorylatable amino acids, it is plausible that phosphorylation of a number of these residues could contribute to a decrease in the antibody-antigen interaction. To reconcile these apparently incongruous results, we propose that the data of Johnson et al. (2003) together with ours suggest that the N-terminal region of TGA2 is either inaccessible to the antibody or that the epitope is post-translationally modified when cells are unstimulated or in the absence of NPR1.

In an in vivo transcription system based on the *PR-1* promoter (Figure 1J), we could demonstrate that, in uninduced cells, TGA2 repressed transcription of the *PR-1* promoter activated only by expression of NPR1 (Figure 1J white bars; NPR1 compared to TGA2 + NPR1). However, TGA2 was unable to repress the baseline level of *PR-1* expression as defined by expression of the unrelated protein Gal4 DB (Figure 1J). Therefore, in the context of *PR-1* and in uninduced cells, TGA2 may only serve to repress the activating effect resulting from the recruitment of NPR1 to the promoter. TGA2:DB can also repress transcription from a LexA:VP16-activated synthetic promoter (Supplemental Figure 2). These data further emphasize the fact that TGA2 is not a transcriptional activator on its own. The repressing effect of TGA2 observed on the *PR-1* promoter activated by NPR1 is likely independent of an interaction between TGA2 and

NPR1, since there is no detectable interaction between these two proteins in the context of the *PR-1* promoter (Figure 1J white bars; TGA2 compared to TGA2 + NPR1:TA). This is further substantiated by the observation that the activating effect resulting from the recruitment of nim1-2 (an NPR1 mutant version that does not interact with TGA2) to *PR-1*, is also repressed by TGA2 (Figure 1J white bars; nim1-2 compared to TGA2 + nim1-2).

3.4.2 NPR1 Is a Transcriptional Co-Activator in SA-Stimulated Cells

When tethered to DNA through the Gal4 DB, NPR1 activates transcription only after cells have been stimulated with SA. However, the finding that expression of NPR1 without fusion to the Gal4 DB does not lead to transcriptional modulation indicates that recruitment to the promoter is required for transcriptional activation. In the absence of a fusion to the Gal4 DB, NPR1 can be recruited to the heterologous Gal4 promoter via TGA2:DB. This recruitment leads to transactivation of the TGA2-NPR1 complex in SA-treated cells (Figure 1H), thus defining NPR1 as a co-activator. Remarkably, the TGA2-NPR1 complex is sufficient to activate the heterologous (Figure 1H) and the *PR-1* promoter (Figure 1J) in an SA-dependent fashion. Therefore, the complex behaves as an SA-regulated enhanceosome exposing a unique activating interface (Merika and Thanos 2001; Thanos and Maniatis 1995). Transactivation of the TGA2-NPR1 enhanceosome requires the core of the NPR1 BTB/POZ domain, since deletion beyond it (Δ110) or its mutation (alanine-substitution), abolishes the function of the enhanceosome both on a transiently-delivered heterologous promoter (Figure 3A) and on the endogenous *PR-1*

gene (Figure 2B). Transactivation of the TGA2-NPR1 enhanceosome is also dependent on the oxidation of C521 and C529 of NPR1 (Figure 5).

Although NPR1 behaves as a transcriptional activator in SA-treated cells when tethered to DNA on the Gal4-based promoter, this may not be the case when NPR1 is recruited to the *PR-1* promoter. First, addition of a strong transactivation domain (VP16) to NPR1 (NPR1:TA) did not lead to further activation of the transiently-delivered *PR-1* promoter when compared to unfused NPR1 transfected alone (Figure 1J). Second, mutation of C521/C529, which abolishes the transactivation properties of NPR1, activated *PR-1* to the same extent as the wild-type NPR1 (Figure 5H). These results could suggest that in the architectural context of the *PR-1* promoter, the *PR-1*-activating effect of NPR1 observed over the baseline level, may be de-repression as opposed to activation; that is to say the effect may be due to chromatin structure modification instead of an active recruitment of the basal transcription machinery by NPR1. The discrepancy between the results observed on the *Gal4*-based and *PR-1* promoters could arise from dissimilarity in the architecture of the two promoters. It could also arise from dissimilarity in the architecture of protein complexes due to allosteric effects of DNA (Lefstin and Yamamoto 1998), since in one case NPR1 interacts with DNA through a heterologous DB, while in the other, NPR1 is recruited to *PR-1* through an unidentified DB or through an unknown DNA-binding protein, itself recruited to *PR-1*. Therefore, although ChIPs demonstrated that NPR1 is recruited to *PR-1* in both resting and SA-treated cells (Figure 1D and E), its role on the uninduced *PR-1* is unclear. However, it seems reasonable to think that NPR1 interacts with *PR-1* as a ready-to-go latent co-activator. This is consistent with the fact that overexpression of NPR1 does not lead to

constitutive *PR-1* expression; transcription still requires activation by SA (Cao et al., 1998). The nature of this switch remains elusive.

It has been proposed that the role of NPR1, in a wild-type plant, is to inactivate the repressing effect of SNI1 on *PR* genes (Li et al., 1999). As such, in the *sni1 npr1-1* double mutant, *PR* gene expression is restored and is inducible. This also led to the proposal that induction of *PR* genes requires the activation of TGA factors in an SA-dependent, but NPR1-independent fashion. However, it is clear from the results presented here, that TGA2, the prototype of the TGA2/5/6 clade, does not display any autonomous transactivation properties whether or not cells are treated with SA, but requires an association with NPR1 to display such activities. Furthermore, the transactivating capacity of the TGA2-NPR1 complex is dependent upon the functionality of a transactivation domain found in the C-terminus of NPR1. Thus, in the case of the *PR-1* gene, it is unlikely that the role of NPR1 is simply to inactivate SNI1. Instead, we propose that the *sni1* mutation, in the *sni1-npr1* double mutant background, might activate pathways that regulate *PR-1* in an NPR1- and TGA2/5/6-independent fashion. Indeed, it has been shown that *PR-1* is regulated in an SA-dependent, but NPR1-independent fashion by transcription factor AtWhy1 (Desveaux et al., 2004). In resting cells, AtWhy1 is held inactive by an inhibitor, which prevents it from binding to DNA. Upon SA-treatment, AtWhy1 is released from the effects of this inhibitor, which allows it to be recruited to its cognate DNA (Desveaux et al., 2004). It is thus possible that SNI1 plays a role in the AtWhy1-dependent pathway leading to *PR-1* induction as opposed to the TGA2-NPR1-dependent pathway. However, in the absence of ChIP data indicating that SNI1 or AtWhy1 are themselves recruited to the *PR-1* promoter, it is unclear whether

their effects on the *PR-1* promoter are direct or indirect. Furthermore, in the absence of data indicating that SNI1 can physically interact with TGA2 or NPR1, it is very difficult to place, with any confidence, this protein in a model of *PR-1* regulation.

3.4.3 Cysteine-Oxidized Transactivation Domain: A New Type of Transactivation Domain

Cysteine residues in eukaryotic transcription factors have been demonstrated to be the target of redox regulation. In most cases cysteines affect DNA-binding activity, which is abolished when these are oxidized (Abate et al., 1990; Lando et al., 2000; Toledano and Leonard 1991). However, in a few instances oxidation has been shown to control homodimerization (Benezra 1994) and to inhibit nuclear export (Kuge et al., 2001). When one eliminates cases where effects on transactivation are due to modulation of DNA-binding activity (as opposed to modulation of transactivation per se), there is only one example in the literature where a transactivation domain is controlled by cysteine redox. However, in this instance, oxidation abolished transactivation (Morel and Barouki 2000). It thus appears that NPR1 is a rare example of a transactivation domain positively regulated by oxidized cysteines (C521 and C529). Remarkably, despite the fact that C521 and C529 are oxidized regardless of whether cells are exposed to SA (Figure 5C), these cysteines only modulate transactivation in SA-stimulated cells (Figure 5B). This suggests that different factors mediating contact between the NPR1 transactivating domain and the basal transcription machinery operate in non-induced and SA-stimulated cells.

The results reported in this paper constitute a significant advancement of our knowledge on plant disease resistance by elucidating the molecular function of TGA2 as a transcriptional repressor of NPR1-dependent de-repression of *PR-1* and of NPR1 as a co-activator of TGA2, and by establishing the existence of an SA-regulated enhanceosome composed of at least TGA2 and NPR1. Figure 6 presents a model that summarizes the results reported in this paper on the regulation of *PR-1*.

3.5 METHODS

3.5.1 Plant Transcription Assays and Two-Hybrid Assays

All procedures for the yeast two-hybrid system were previously described (Després et al., 2000). All procedures for the plant two-hybrid assays, the reporter gene vector, the internal standard vector, and the VP16:NPR1 construct were previously described (Després et al., 2003). *TGA2*, *NPR1*, the alanine-substitution and deletion mutants of *NPR1* were created by PCR using appropriate primers and cloned in-frame with the GAL4 DB or VP16 TA contained in pBI524, respectively, to create N-terminal fusion proteins as described (Després et al., 2003). The unfused versions of *TGA2* and *NPR1* were cloned into pBI524 lacking the GAL4 DB or VP16 TA. To create the *PR-1* promoter-luciferase reporter gene fusion, the -1293 promoter fragment (Lebel et al., 1998) was amplified by PCR and used to substitute the *5X UAS*^{GAL4} fragment in the luciferase-*nopaline synthase* (*nos*) polyadenylation signal reporter plasmid. Every bar in

each graph represents five bombardments repeated five times (n = 25). *Arabidopsis thaliana* ecotype Columbia was used throughout this study.

3.5.2 Chromatin Immunoprecipitation (ChIP) of NPR1

ChIP was performed as we described previously (Chakravarthy et al., 2003). The specificity of the anti-NPR1 antibody has been demonstrated previously (Després ate al., 2000). The agarose-conjugate Gal4 DB antibody was from Santa Cruz Biotechnology (Santa Cruz, CA; sc-510 AC). The PCR primer pair specific to the *PR-1* promoter is as follows: 5'-ATGGGTGATCTATTGACTGTTT-3' and 5'-GTAGCTTTGCCATTGTTGAT-3'. To confirm that the PCR product generated was indeed a fragment of the *PR-1* promoter, it was gel excised, cloned, and sequenced.

3.5.3 Plant Growth Conditions and Transformation

Conditions for growth of *Arabidopsis thaliana* (Columbia) and *npr1-3* plants (Cao et al., 1997) and methods for plant transformation, the selection of transgenic individuals, and northern blot hybridization were previously described (Liu et al., 2005).

3.5.4 In Vivo Determination of the Cys Redox Status of NPR1 Δ513

Due to very low amounts of proteins in the biolistics assays, 80 bombardments were performed with the Δ513:TA constructs. After a 24-hr incubation period with or without SA, proteins were extracted from *Arabidopsis thaliana* leaves, separated into two aliquots and processed immediately and in parallel as described previously (Després et al., 2003). Immunoprecipitations were performed with an anti-VP16 antibody (sc-7545 AC, Santa Cruz Biotechnology, Ca). The VP16 TA does not contain any cysteines.

3.5.5 Statistical Methods

All pooled data are expressed as averages and error bars represent ± 1 standard deviation. When data from two independent populations are compared, statistical significance was assessed using a two-tailed Student *t*-test.

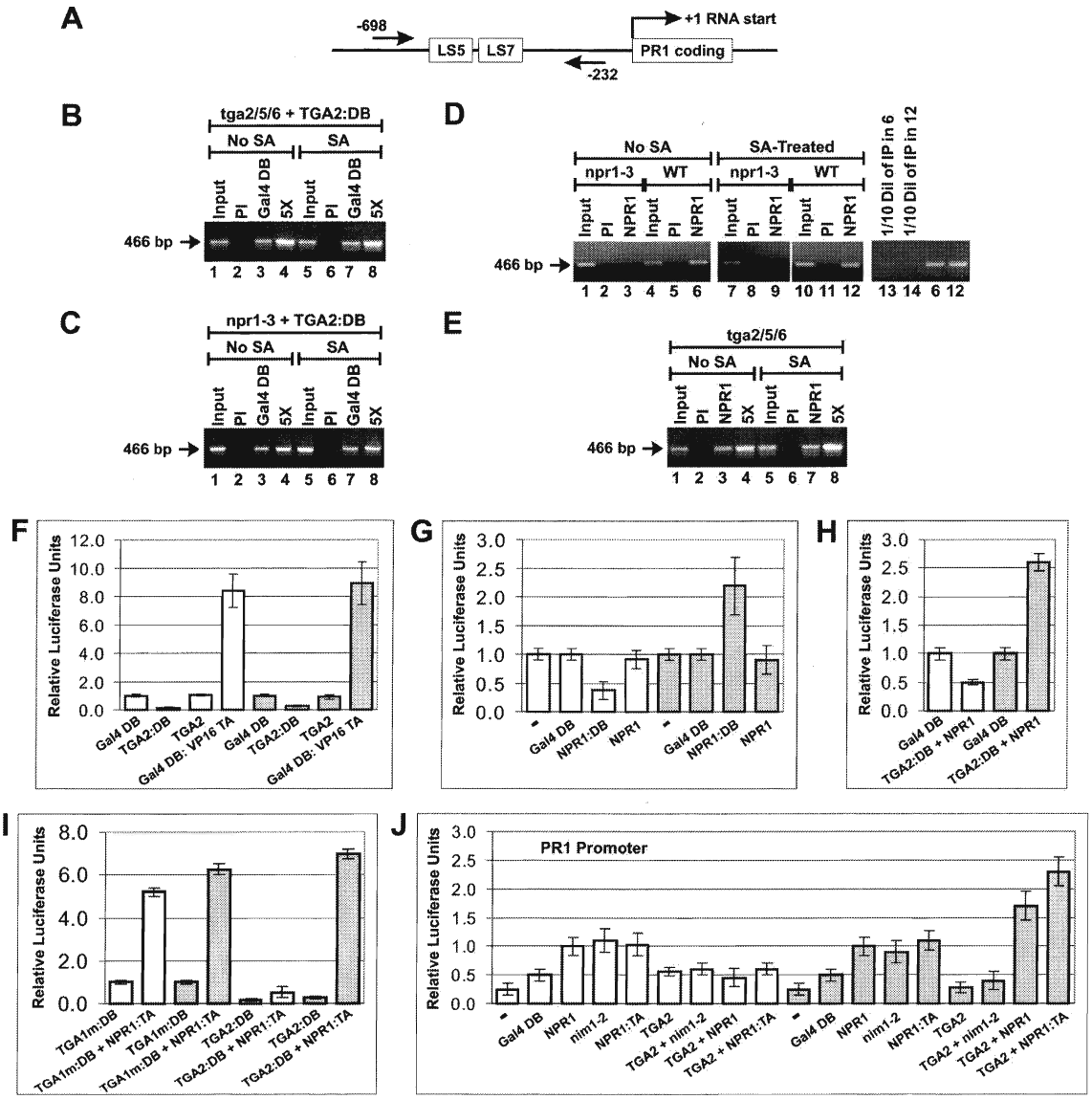


Figure 1. NPR1 Is a Co-Activator Required for Transcriptional Activation by a TGA2-NPR1 Complex in SA-Treated Cells Only.

(A) Graphic representation of the *PR-1* gene. The straight arrows and the numbers indicate the position of the PCR primers used for ChIP experiments. LS5 and LS7 are two DNA regions containing the TGA factors cognate binding sequence TGACG (Lebel et al., 1997).
(B) Chromatin Immunoprecipitations of TGA2:DB expressed in tga2/5/6 knock-out or **(C)** *npr1-3* mutant (npr1-3) *Arabidopsis* plants were conducted with anti-Gal4 DB antibodies conjugated to agarose beads.
(D) Chromatin Immunoprecipitations of NPR1 from wild-type (NPR1), *npr1-3* mutant (npr1-3) or **(E)** tga2/5/6 knock-out *Arabidopsis* plants were conducted with anti-NPR1 antibodies. The specificity of the anti-NPR1 antibody has been demonstrated previously (Després ate al., 2000).

In **(B)**, **(C)**, **(D)**, and **(E)** tissues were untreated (No SA) or treated for 6 hr with 1 mM SA. PI indicates that ChIP was performed with pre-immune serum. PCR was conducted with *PR-1* promoter-specific primers. The arrow indicates the location of the PCR products. The NPR1-3 protein is a deletion version of NPR1 (Cao et al., 1997), which has lost the antigenic region used to raise the anti-NPR1 antibodies used in this study. The inputs represent 2% of the immunoprecipitated material (50-fold dilution). 5X indicate that the PCR reaction was performed with 5 times the amount of immunoprecipitated material, to demonstrate that the PCR reaction was in the linear range. In lanes 13 and 14 of panel (D), 1/10 of the amount of immunoprecipitated material used in lanes 6 and 12, respectively, was used to perform the PCR to demonstrate that the PCR reaction was in the linear range.

(F) Histograms illustrating the fact that TGA2 tethered to DNA through Gal4 DB (TGA2:DB) does not activate transcription, while a chimeric transcription activator composed of the Gal4 DB fused to the transactivation domain of viral particle 16 (Gal4 DB:VP16 TA) does. Gal4 DB represents the baseline level of transcription.

(G) Histograms illustrating the transcription activation of NPR1 tethered to DNA through Gal4 DB (NPR1:DB). NPR1 indicates the absence of fusion. (-) indicates that only the reporter and internal standard vectors have been bombarded into the tissues; no effector has been introduced.

(H) Histograms illustrating the effect of NPR1 on the transcriptional activity of TGA2:DB. NPR1 indicates that the protein is expressed without a fusion.

(I) Histograms illustrating the fact that TGA2 tethered to DNA through Gal4 DB (TGA2:DB) interacts very poorly with NPR1:TA in the absence of SA-treatment.

In **(F)**, **(G)**, **(H)**, and **(I)** *Arabidopsis* leaves were left untreated (white bars) or were treated for 24 hr with 1 mM salicylic acid (grey bars). The constructs were transfected along with the *5X UAS*^{GAL4}:Firefly luciferase reporter and the *CaMV35S*:Renilla luciferase internal standard vectors. Data are reported as Relative Luciferase Units. The fold-activation represents the Relative Luciferase Units (RLU) obtained with the given protein or protein pair divided by the RLU obtained with the unfused Gal4 DB construct alone (baseline transcription). Values consist of n=25 samples and represent averages ± 1 SD. Every bar represents five bombardments repeated five times (n = 25).

(J) Histogram illustrating the effect of NPR1, and nim1-2 on the transcriptional activity of the TGA2-NPR1 complex. All proteins were native (without fusion), with the exception of NPR1:TA (NPR1 fused to the viral particle 16 transactivation domain), which was used to assess the level of interaction between NPR1 and TGA2 in the context of the *PR-1* promoter. The reporter system was the *Arabidopsis PR-1* promoter fused to the firefly luciferase. The *CaMV35S* promoter:Renilla luciferase fusion was used as an internal standard. (-) indicates that no effector were bombarded along with the reporter and internal standard vectors. *Arabidopsis* leaves were left untreated (white bars) or were treated for 24 hr with 1 mM salicylic acid (grey bars). Data are reported as Relative Luciferase Units. Values consist of n=25 samples and represent averages ± 1 SD. Every bar represents five bombardments repeated five times (n = 25).

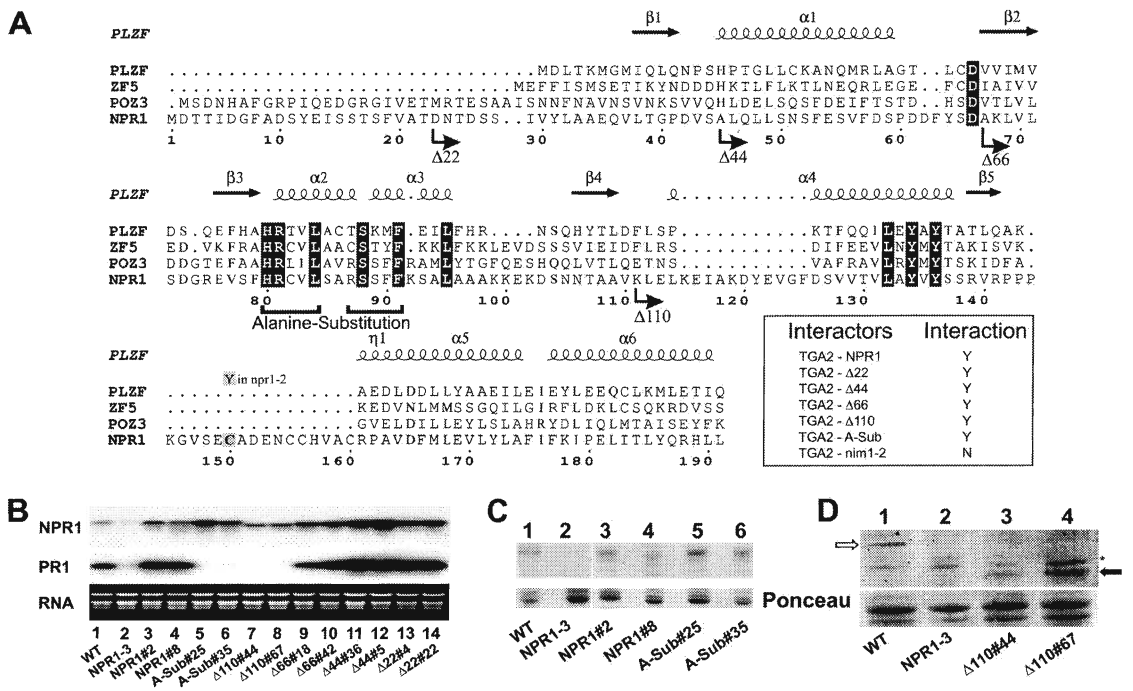


Figure 2. The BTB/POZ Domain of NPR1 Is Required for *PR-1* Induction.

(A) Multiple alignment of selected BTB/POZ domains. Residues blocked in black are conserved among all sequences. Numbers refer to the amino acid position in NPR1. Straight arrows and coils indicate the position of beta strands and helices in the PLZF crystal structure, respectively. α and η indicates α- and 3_{10}- helices, respectively and β refers to β-strands. The bent arrow indicates the position where the NPR1-deletion proteins begin. The horizontal brackets below the amino acid sequence of α2 and α3 indicate the residues that have been mutated to alanine in the "Alanine-Substitution" mutant. C150 bears a C to Y mutation in the npr1-2 mutant, which abolishes interaction with TGA2, *PR* gene activation, and deployment of SAR (Cao et al., 1997; Després et al., 2000; Zhang et al., 1999). NPR1, PLZF, POZ3, and ZF5 are from Genbank accession numbers GI:1773295, GI:486933, GI:2291257, and GI:1399185, respectively. The inset represents directed yeast two-hybrid assays using the filter test and the outcome of the experiments. nim1-2 is a mutant version of NPR1 that bears a histidine-to-tyrosine replacement in one of the ankyrin repeats (Ryals et al., 1997), which abolishes interaction with TGA factors (Després et al., 2000 and 2003). Y (yes), indicates an interaction while N (no), indicates an absence of interaction (white color after 24 hr incubation with X-GAL).

(B) Northern blot analysis using *NPR1* or *PR-1* probes. RNA stained with ethidium bromide is shown for loading comparison. Lane 1 contains RNA from wild-type *Arabidopsis* and lane 2 from the *npr1-3* mutant. The remaining lanes contain RNA from *npr1-3* lines expressing the following constructs; wild-type NPR1 (Lanes 3 and 4), the "Alanine-Substitution" mutant (Lanes 5 and 6), and the deletion mutants Δ110 (Lanes 7 and 8), Δ66 (Lanes 9 and 10), Δ44 (Lanes 11 and 12), and Δ22 (Lanes 13 and 14). Results from two independent transgenic lines are shown per construct. Specific line numbers follow the construct name.

(C-D) Top Panel. Immunoblot analysis of proteins from wild-type *Arabidopsis* (WT), the *npr1-3* mutant (NPR1-3), and the *npr1-3* background lines expressing NPR1, the "Alanine-Substitution" mutant and Δ110 as described in **(B)**. An anti-NPR1 antibody (Després et al., 2000) was used. Bottom Panel. Ponceau staining of the membranes shown in the top panel. In panel **(D)**, the open arrow indicates the position of the full-length NPR1 protein (66 kD), while the black arrow indicates that of the truncated protein Δ110 (54.4 kD). The asterisk points to a protein interacting non-specifically with the antibody.

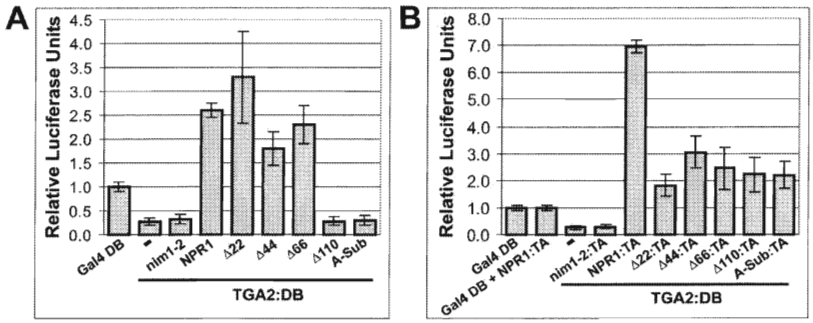


Figure 3. The Core of the NPR1 BTB/POZ Is Required for the TGA2-Dependent Co-Activator Function of NPR1 in SA-Treated Cells.

(A) Histogram illustrating the effect of NPR1 and the mutants described in Figure 2, on the transcriptional activity of the TGA2-NPR1 complex tethered to DNA through Gal4 DB fused to TGA2. Results obtained with TGA2:DB alone (-) are also shown.
(B) Histogram illustrating the interaction of NPR1 and the mutants described in **(A)** fused to the VP16 transactivation domain with TGA2 fused to the Gal4 DB. Results obtained with Gal4 DB alone (Gal4 DB), Gal4 DB coexpressed with NPR1:TA (Gal4 DB + NPR1:TA), and TGA2:DB alone (-) are also shown. For **(A)** and **(B)** conditions were identical to those described in Figure 1. Data are reported as Relative Luciferase Units. Values consist of n=25 samples and represent averages ± 1 SD. Every bar represents five bombardments repeated five times (n = 25).

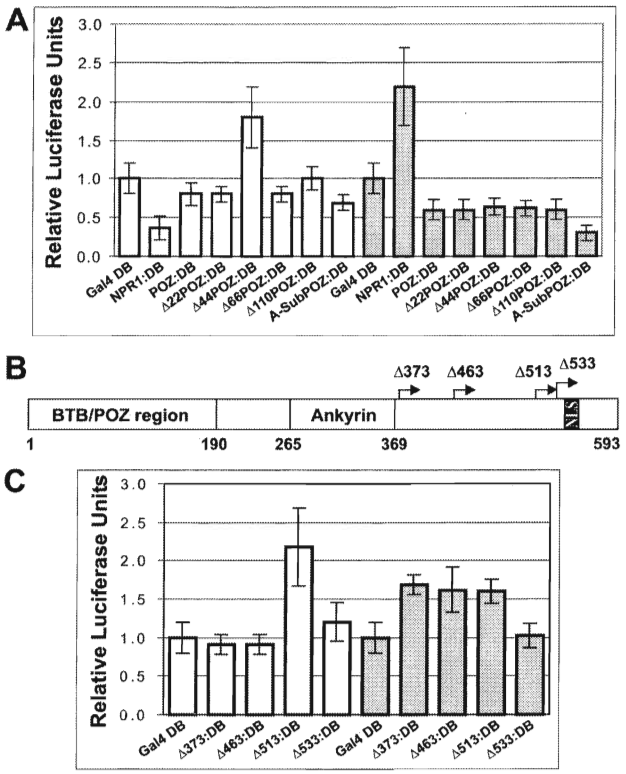


Figure 4. NPR1 Harbors an Autonomous Transactivation Domain in the last 80 Residues.

(A) Histogram illustrating the transcriptional activity of the NPR1 BTB/POZ domain, the deletion mutants of the BTB/POZ, and the Alanine-Substitution mutant tethered to DNA through Gal4 DB. The deletion and the Alanine-Substitution mutants were created starting with the NPR1 BTB/POZ domain. BTBPOZ region represents the first 190 amino acids of NPR1.

(B) Schematic representation of NPR1 and the deletions analyzed in panel **(C)**. The numbers preceded by Δ indicate the starting amino acid for the particular deletion mutant. NLS indicates the nuclear localization signal. Ankyrin represents the region containing the ankyrin repeats as defined by Pfam and SMART. Diagram is drawn to scale.

(C) Histogram illustrating the transcriptional activity of the NPR1 deletion mutants described in **(B)**, tethered to DNA through Gal4 DB. For **(A)** and **(C)** conditions were identical to those described in Figure 1. Data are reported as Relative Luciferase Units. Values consist of n=25 samples and represent averages ± 1 SD. Every bar represents five bombardments repeated five times (n = 25).

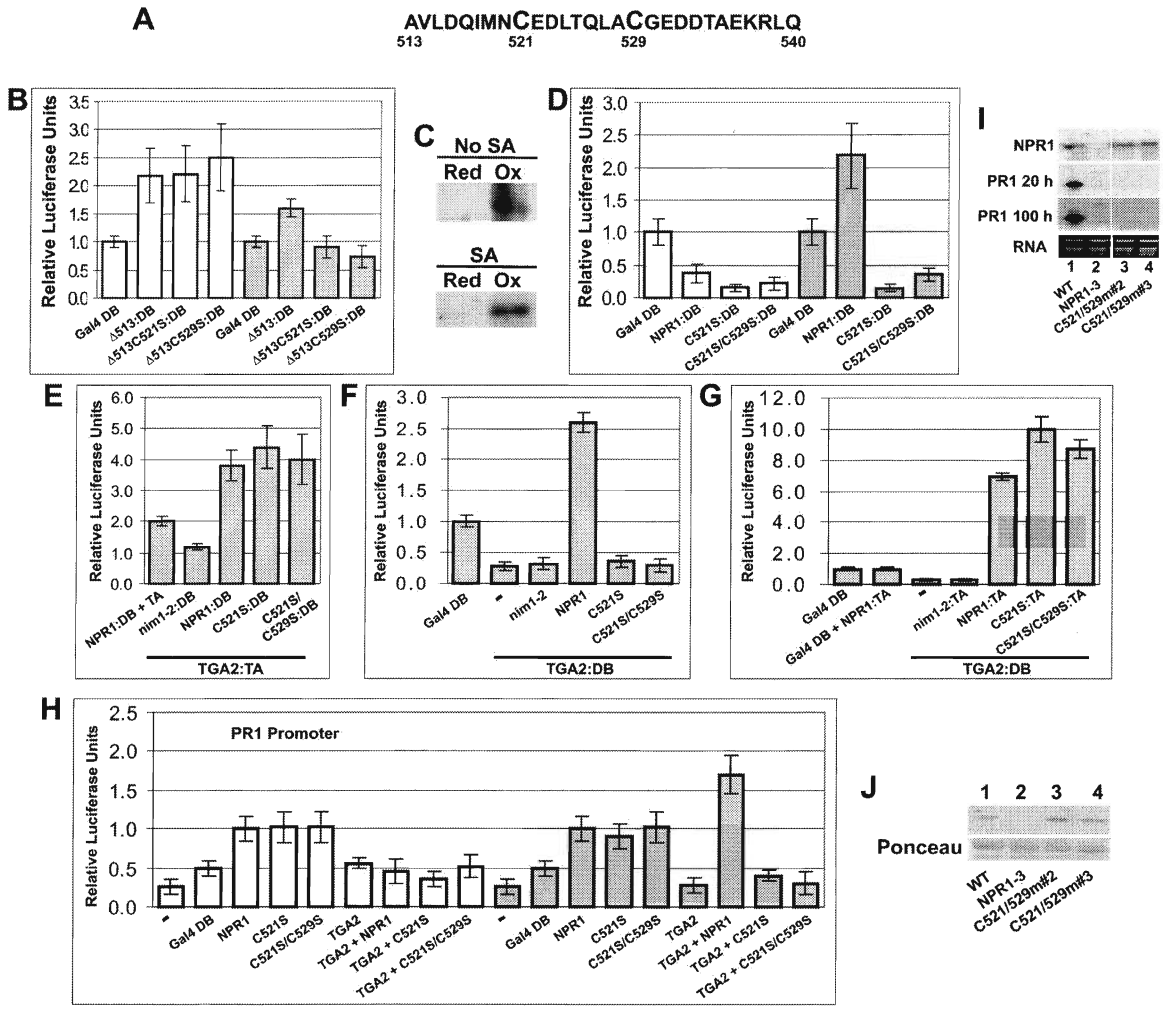


Figure 5. Oxidation of C521 and C529 Correlates with Transcriptional Activation of the *PR-1* Gene by the TGA2-NPR1 Complex.

(A) Sequence of amino acids located between position 513 and 540.

(B) Histogram illustrating the transcriptional activity of the Δ513 deletion mutant of NPR1 and the effect of mutating C521 or C529, within the context of the Δ513 protein. Proteins were tethered to DNA through Gal4 DB.

(C) Blot analysis of NPR1Δ513 immunoprecipitate used to assess the in vivo redox status of residues C521 and C529 present in cells of *Arabidopsis* leaves treated for 24 hr with SA (SA) or left untreated (No SA). Red indicates immunoprecipitates from proteins labeled for reduced Cys residues, while Ox indicates immunoprecipitates from proteins labeled for oxidized Cys (see Experimental Procedures).

(D) Histogram illustrating the transcriptional activity of the full-length NPR1 and the effect of mutating C521 or simultaneously C521 and C529, within the context of the full-length NPR1. Proteins were tethered to DNA through Gal4 DB.

(E) Histogram illustrating the interaction of NPR1, the C521, or the C521 and C529 mutants described in **(D)**, with TGA2 fused to the VP16 transactivation domain. nim1-2, which does not interact with TGA2, was also expressed with TGA2:DB as a negative

control. NPR1, nim1-2 and the mutants described in **(D)** were all fused to the Gal4 DB. NPR1:DB was also expressed along with the VP16 transactivation domain (NPR1:DB + TA) as another negative control.

(F) Histograms illustrating the effect of NPR1 the C521, or the C521 and C529 mutants described in **(D)** on the transcriptional activity of TGA2:DB. All proteins, except TGA2:DB were expressed without a fusion.

(G) Histogram illustrating the interaction of NPR1, the C521, or the C521 and C529 mutants described in **(D)** all fused to the VP16 transactivation domain, with TGA2 fused to the Gal4 DB.

For **(B)**, **(D)**, **(E)**, **(F)** and **(G)** conditions were identical to those described in Figure 1. Data are reported as Relative Luciferase Units. Values consist of n=25 samples and represent averages ± 1 SD. Every bar represents five bombardments repeated five times (n = 25).

(H) Histogram illustrating the effect of NPR1, nim1-2, and the C521, or the C521 and C529 mutants, on the transcriptional activity of the TGA2-NPR1 complex. All proteins were native (without fusion). The reporter system was the *Arabidopsis PR-1* promoter fused to luciferase. The CaMV 35S promoter:Renilla luciferase fusion was used as an internal standard. (-) indicates that no effector were bombarded along with the reporter and internal standard vectors. *Arabidopsis* leaves were left untreated (white bars) or were treated for 24 hr with 1 mM salicylic acid (grey bars). Data are reported as Relative Luciferase Units. Values consist of n=25 samples and represent averages ± 1 SD. Every bar represents five bombardments repeated five times (n = 25).

(I) Northern blot analysis using *NPR1* or *PR-1* probes. RNA stained with ethidium bromide is shown for loading comparison. Lane 1 contains RNA from wild-type *Arabidopsis* and lane 2 from the *npr1-3* mutant. Lanes 3 and 4 contain RNA from two independent *npr1-3* transgenic lines expressing NPR1 bearing cysteine-to-serine mutations at position 521 and 529. Specific line numbers follow the construct name. PR1 20 h and PR1 100h represent a 20-hour and 100-hour autoradiography, respectively. All lanes are from the same gel or immunoblot.

(J) Top Panel. Immunoblot analysis of proteins from wild-type *Arabidopsis* (WT), the *npr1-3* mutant (NPR1-3), and the *npr1-3* background lines expressing the mutant described in **(I)**. An anti-NPR1 antibody (Després et al., 2000) was used. Bottom Panel. Ponceau staining of the membranes shown in the top panel.

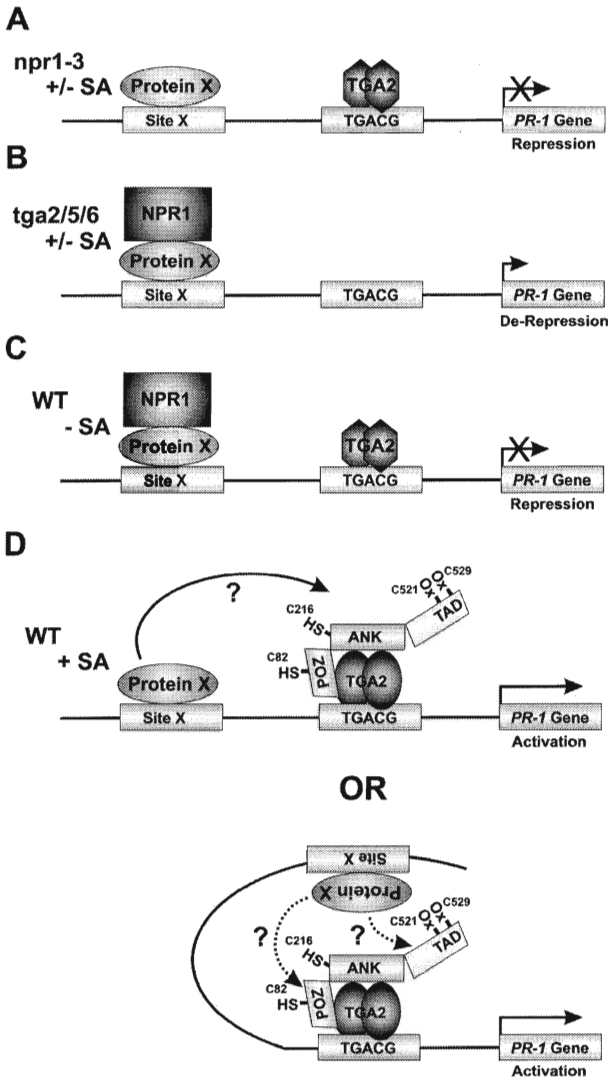
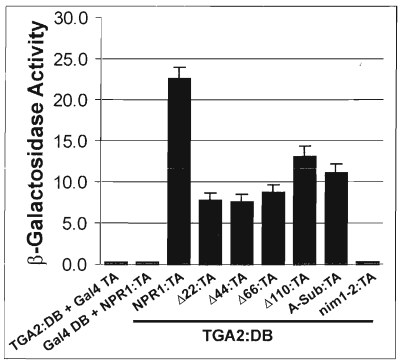


Figure 6. Working Model for Regulation of *PR-1* by the TGA2-NPR1 Enhanceosome.

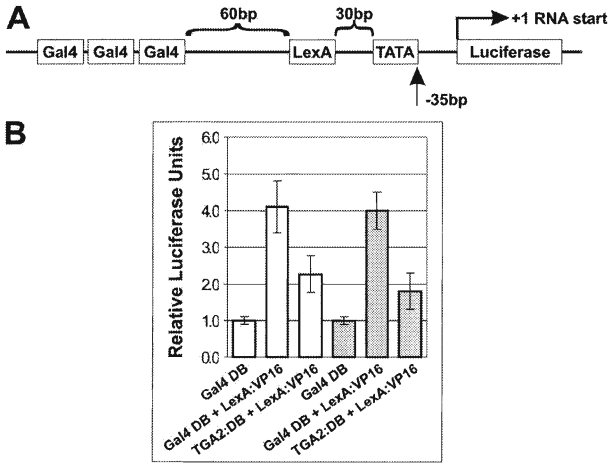
(A) In an *npr1* mutant plant such as *npr1-3*, there is no NPR1-dependent de-repression of *PR-1* and there is no incorporation of TGA2 into a TGA2-NPR1 enhanceosome. *PR-1* is repressed. Since NPR1 is recruited to *PR-1* independently from TGA2 and since NPR1 does not contain a known DNA binding domain, we postulate that in a wild-type plant NPR1 is recruited through an unknown protein (Protein X) binding to an unknown DNA element (Site X).
(B) In the *tga2/5/6* triple knock-out (Zhang et al., 2003), NPR1 is recruited to the *PR-1* promoter which becomes de-repressed. In these plants, the TGA2-NPR1 enhanceosome is not recruited to the *PR-1* promoter, due to the absence of TGA2, TGA5, and TGA6.
(C) In a wild-type plant unstimulated with SA, both NPR1 and TGA2 are recruited to the *PR-1* promoter independent of each other. However, under resting condition, NPR1 and TGA2 do not interact with each other. Again here, NPR1 is postulated to be recruited through an unknown protein (Protein X).

(D) In the presence of SA, NPR1 forms an enhanceosome with TGA2. Transactivation of the complex requires the oxidation of C521 and C529, which are found within the confines of a transactivation domain (TAD) in the C-terminus of NPR1. Of note, C82 and C216 must be reduced for NPR1 to monomerize (Mou et al., 2003). The BTB/POZ domain of NPR1 is hypothesized to interact with TGA2. In the top panel, NPR1 is postulated to be transferred from the unknown Protein X to TGA2. In the bottom panel, NPR1, Protein X, and TGA2 are postulated to interact all at the same time. Protein X does not interact with NPR1 through the ankyrin repeats and is proposed to interact with the N-terminus or the C-terminus of NPR1 or both. It is thus clear that the nature of the enhanceosome remains undetermined, but it contains at the very least NPR1 and TGA2.



Supplemental Figure 1. The BTB/POZ Mutants of NPR1 Interact with TGA2 in Yeast Two-Hybrid Assays.

Quantitative yeast two-hybrid assays illustrating the interaction between TGA2 produced as a Gal4DB fusion (TGA2:DB) co-expressed with NPR1, nim1-2, NPR1 lacking the first 22, 44, 66, or 110 amino acids (Δ22, Δ44, Δ66, Δ110, respectively), or NPR1 mutated in the core of the BTB/POZ domain (A-Sub) expressed as Gal4TA fusions (:TA). TGA2:DB co-expressed with Gal4TA and Gal4DB co-expressed with NPR1:TA served as negative controls. Values represent averages ± 1 SD.



Supplemental Figure 2. TGA2 Represses a LexA:VP16-activated Synthetic Promoter.

(A) Graphic representation of the synthetic *3X Gal4:1X LexA:minimal promoter:Firefly Luciferase* reporter gene. The upward arrow indicates the position of the TATA box relative to the RNA start site. 60bp and 30 bp indicate the spacing in base pairs between the most downstream Gal4 element and the LexA element and between the LexA element and the TATA box, respectively. Not shown is an omega translational enhancer in the transcribed region of the Luciferase gene.

(B) Histograms illustrating the fact that TGA2 tethered to DNA through Gal4 DB (TGA2:DB) represses transcription of a LexA DB fused to the transactivation domain of viral particle 16 (LexA:VP16) only when cells are treated with SA. *Arabidopsis* leaves were left untreated (white bars) or were treated for 24 hr with 1 mM salicylic acid (grey bars). The constructs were transfected along with the *3X* UAS^{GAL4}*:1X LexA DNA element:minimal promoter:*Firefly luciferase reporter and the *CaMV35S*:Renilla luciferase internal standard vectors. Data are reported as Relative Luciferase Units. Values consist of n=25 samples and represent averages ± 1 SD. Every bar represents five bombardments repeated five times (n = 25).

CHAPTER 4 – The Transcriptional Activator Pti4 Is Required for the Recruitment of a Repressosome Nucleated by Repressor SEBF at the Potato *PR-10a* Gene

Contributions

This manuscript was the product of a secondary research project I conducted in collaboration with the lab of Dr. Normand Brisson at the University of Montreal. I was invited to participate in this project due to my expertise with both the plant two hybrid assay and ChIP techniques. My contributions to this document include Figure 4: A-F, Figure 5: A and B, Figure 6: B and Supplemental Figures 2 and 3. I was directly involved in developing and testing hypotheses that were critical to the manuscript. I also contributed to the writing of the discussion and the editing of the entire manuscript.

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4.1 ABSTRACT

Transcriptional reprogramming is critical for plant disease resistance responses. In potato, the marker gene *PATHOGENESIS-RELATED-10a* (*PR-10a*) is transcriptionally activated by pathogens, wounding or elicitor treatment. Activation of *PR-10a* requires the recruitment of the activator StWhy1 to its promoter. In addition, *PR-10a* is negatively regulated by the repressor SEBF. Here, we show through a yeast two-hybrid screen that SEBF interacts with the transcriptional activator Pti4. SEBF recruits Pti4 via its cs-RBDI RNA binding domain, while Pti4 is recruited to SEBF by means of its ERF domain. In vivo plant transcription assays confirmed that SEBF interacts with Pti4 to form a repressosome, despite the fact that Pti4 is a transcriptional activator. Chromatin immunoprecipitation revealed that both SEBF and Pti4 are recruited to the *PR-10a* promoter in uninduced conditions only and that the recruitment of Pti4 was dependent on the presence of SEBF, consistent with the fact that there is no GCC-box in *PR-10a*. Unexpectedly, we also demonstrated that recruitment of SEBF was dependent on the presence of Pti4, rationalizing the reason why SEBF, itself a repressor, requires Pti4 for its repressing function. The data presented here constitutes a paradigm-shift of repressosome function in plants.

4.2 INTRODUCTION

To combat the invasion of potential pathogens, plants possess an immune system with which they detect elicitors and activate a battery of defense responses. As a result of massive transcriptional reprogramming, the spread of pathogens can be stopped and, in some cases, a plant could become resistant to subsequent invasions (Jones and Dangl, 2006). Plant defense mechanisms are linked to the up-regulation of *Pathogenesis-Related* (*PR*) gene expression as well as other responses, such as the production of antimicrobial compounds and modification of secondary cell wall composition (Stintzi et al., 1993). However, *PR* genes represent only a subset of the large number of genes whose expressions are modified in response to pathogen attack. In the model plant *Arabidopsis*, for example, up to 25% of all the genes are subjected to changes in regulation (Eulgem, 2005). Therefore transcription factors fulfill a crucial role in the regulation of plant defense responses.

Several families of transcription factors involved in regulating plant defense have been characterized, including WRKY, Myb, ERF, Whirly and TGA transcription factors (Fobert, 2006). Members of the ethylene-response factors (ERF) bind the GCC box found in the promoter of many defense-related genes (Ohme-Takagi and Shinshi, 1995; Ohme-Takagi et al., 2000). ERF transcription factors are regulated by ethylene, jasmonic acid (JA), salicylic acid (SA) and some pathogen infections (Brown et al., 2003; Lorenzo et al., 2003; Oñate-Sánchez and Singh, 2002; Gu et al., 2000) as well as by abiotic stresses (Chen et al., 2002; Park et al., 2001). The tomato Pti4 is an ERF transcription factor that was first isolated by its interaction with the kinase Pto, which confers resistance to

Pseudomonas syringae pv. *tomato* expressing the avirulence gene *AvrPto* (Zhou et al., 1997). Pti4 controls the expression of defense-related genes and its function is regulated at both the transcriptional and post-transcriptional levels (Gu et al., 2000; Mysore et al., 2002, Wu et al., 2002). By virtue of its ERF domain, Pti4 can bind the sequence GCCGCC (GCC-box) and regulate the expression of several GCC-box-containing genes (Gu et al., 2002). However, chromatin immunoprecipitation (ChIP) experiments have shown direct binding of Pti4 to some non-GCC-box-containing promoters (Chakravarthy et al., 2003), leading to the hypothesis that either Pti4 is able to bind to a DNA motif other than the GCC-box, or that it interacts with other transcription factors to regulate promoter activity (Chakravarthy et al., 2003).

The promoter of the potato *PR-10a* gene has been used as a model to understand defense-related transcriptional regulation. Several regulatory elements were characterized in this promoter, including an elicitor response element (ERE) that confers wounding- and elicitor-dependent transcriptional up-regulation of *PR-10a* (Matton et al., 1993; Després et al., 1995). The recruitment of the transcriptional activator StWhy1 (formerly PBF-2) to the ERE is required for the activation of *PR-10a* (Desveaux et al., 2000). In un-stimulated cells, StWhy1 is stored inactive and sequestered away from the ERE (Desveaux et al., 2000). Upon elicitation, the DNA-binding activity of StWhy1 is induced, allowing the recruitment of the protein to the ERE (Desveaux et al., 2000; Desveaux et al., 2004). *PR-10a* transcription is also regulated through another promoter sequence, located between positions -52 and -27, called the silencer element (SE). Binding of the transcription factor SEBF (Silencing Element Binding Factor) to the SE represses *PR-10a* expression (Boyle and Brisson, 2001; Matton et al., 1993; Després et

al., 1995). Like StWhy1, SEBF is also a single-stranded DNA-binding protein. In addition, SEBF possesses a transit peptide capable of targeting the protein to the chloroplast (Boyle and Brisson, 2001). The mature protein, which is found in plastids and in the nucleus, contains two consensus sequence-type RNA-binding domains (cs-RBDs) separated by a glycine-rich region. (Boyle and Brisson, 2001).

Here we report on the interaction between the repressor SEBF and the potato homolog of the tomato transcriptional activator Pti4. We demonstrate that SEBF interacts with the SE of *PR-10a* through its cs-RBDII, but recruits Pti4 via its cs-RBDI. We show that Pti4 is recruited to SEBF by means of its ERF domain. We also show that SEBF associates with *PR-10a* in un-stimulated cells only and serves to draft Pti4 to the *PR-10a* promoter, which contains no GCC box. We provide evidence that the binding of SEBF to the promoter requires the presence of Pti4 and that the SEBF-Pti4 complex forms the core of a repressosome. The data presented here not only unravel an unprecedented and unexpected role for the activator Pti4 as an indispensable element of a repressosome, but also provide concrete evidence for the previously hypothesized mechanism of recruitment of Pti4 to non-GCC-box-containing genes.

4.3 RESULTS

4.3.1 SEBF Physically Interacts with Pti4 in Yeast and In Vitro

Since SEBF is one of the rare examples of a single-stranded DNA binding repressor characterized from plant systems, we sought to determine the protein composition of the

SEBF-containing repressosome complex. To do so, a cDNA encoding the mature form of the potato SEBF was used as bait in a yeast two-hybrid screen against a tomato cDNA library (Zhang et al., 1999). From ~10^7 transformants, 80 colonies producing blue color on X-gal plates and capable of growth on medium lacking histidine, tryptophan, leucine, and uracil, but supplemented with galactose were identified. Three of these colonies encoded tomato Pti4 (SlPti4). Pti4 is a transcription factor involved in plant defense signaling (Gu et al., 2000) and was chosen for further studies. The full-length coding region of potato Pti4 was amplified from a potato cDNA library (Matton and Brisson, 1989). It codes for a 26 kD protein, which is 94% identical to SlPti4 (Supplemental Figure 1). Potato *Pti4* (*StPti4*, from here on referred to as *Pti4*) also interacted with SEBF (Figure 1A) and was used for all subsequent experiments.

To confirm the yeast two-hybrid results and the direct physical interaction between SEBF and Pti4, we performed pull-down assays. SEBF fused to a C-terminal 6-histidine tag was produced in *E. coli* and coupled to a nickel column before incubation with the C-terminal HA-tagged Pti4 produced in yeast. Analysis of the bound fraction by immunoblot reacted with anti-HA antibodies, as presented in Figure 1B, revealed the presence of a signal (lane 4), demonstrating the existence of an in vitro interaction between Pti4 and SEBF. However, when Pti4-HA was incubated with the resin alone (lane 3) or when only a yeast extract was incubated with His-tagged SEBF bound to the column (lane 2), no signal was detected on the immunoblot, testifying to the specificity of the interaction.

4.3.2 SEBF Recruits Pti4 through cs-RBDI and Interacts with DNA through cs-RBDII

To gain some insights into the protein-protein interaction interface existing between SEBF and Pti4, we performed yeast two-hybrid assays with a series of SEBF deletions fused to the LexA DB, as depicted in Figure 2A, and full-length Pti4 fused to the B42 TA domain. Co-expression of Pti4 with SEBF-1, 2, and 3, which removed, respectively, the basic domain, cs-RBDII, and the glycine-rich domain, did not abolish β-galactosidase activity, indicating that these domains of SEBF were not required for interaction with Pti4 (Figure 2B). Of note, however, deletion of cs-RBDII in SEBF-2 and SEBF-3 stimulated the interaction with Pti4, when compared to that observed with full-length SEBF, suggesting that this domain could be a negative regulator of the interaction. Further deletion, removing cs-RBDI (SEBF-4), reduced reporter gene activity to that observed with the empty vector (pEG202) expressing the DB only. This indicates that Cs-RBDI is required for interaction with Pti4. Consistent with this observation, constructs SEBF-6 and SEBF-7, which lack this domain, did not interact with Pti4.

In the context of full-length SEBF, deletion of the acidic domain (SEBF-5) abolished interaction with Pti4, indicating that this domain might contact Pti4 directly. The acidic domain alone (SEBF-4) was, nevertheless, not sufficient to confer interaction with Pti4. When comparison is made between SEBF-8 and SEBF-3 or between SEBF-9 and SEBF-2, the data seem to indicate that the acidic domain does play a role in the interaction with Pti4. However, in the context of a C-terminal-deleted SEBF (SEBF-8, 9, and 10), the acidic domain did not appear to play an interacting role when compared to SEBF. This ambiguity suggests that the acidic domain of SEBF may be interfacing directly with Pti4, or that it might rather serve to better expose cs-RBDI, which on its

own (SEBF-8), is sufficient for the interaction with Pti4. In Figure 2C, the SEBF constructs were expressed with the empty TA vector to monitor the intrinsic level of reporter activation conferred by these proteins. The levels of reporter gene activity was not significant when compared to those observed when Pti4 was co-expressed (Figure 2B), validating the conclusions that SEBF and Pti4 interact with each other. The immunoblot presented in Figure 2D demonstrates that the lack or low reporter gene activity observed when co-expressing SEBF-4, 5, 6, or 7 with Pti4 was not the result of the absence of expression of these proteins in yeast, but truly reflects a lack of interaction.

Since SEBF possesses two consensus RNA-binding domains (Boyle and Brisson, 2001), we sought to determine which one or whether both of them are required for the single-stranded DNA binding activity. EMSAs represented in Figure 2E and performed with full-length mature SEBF demonstrated a shift indicating binding to the SE-DNA (lane 2). However, deletion of cs-RBDII (SEBF-2) abolished DNA binding (lane 3). Expression of cs-RBDI and cs-RBDII followed by EMSA demonstrated that cs-RBDII (lane 4) was sufficient and was the only domain required for DNA binding activity.

4.3.3 Pti4 Is Recruited to SEBF through its ERF Domain

To further characterize the Pti4-SEBF interaction interface, we performed again yeast two-hybrid assays, but this time using full-length mature SEBF fused to the LexA DB and a series of Pti4 deletions fused to the B42 TA domain, as depicted in Figure 3A. Co-expression of SEBF with Pti4 deletions that progressively removed the N-terminus up to the ERF domain (Pti4-1, 2, and 3), did not substantially alter β-galactosidase activity,

indicating that these domains of Pti4 were not required for interaction with SEBF (Figure 3B). However, a further deletion, removing the ERF (Pti4-4), abolished interaction with SEBF, revealing the importance of this domain for the Pti4-SEBF complex formation. Deleting the C-terminus of construct Pti4-1 up to the ERF (Pti4-5) did not alter the interaction with SEBF, further substantiating the fact that regions outside the ERF are not required for interfacing with SEBF. Attempts to demonstrate that the ERF alone (Pti4-6) was sufficient for interaction with SEBF failed. However, the immunoblot of Figure 3C (lane 7) indicate that Pti4-6 did not express well suggesting that its low abundance may be the explanation for the apparent lack of interaction with SEBF. We thus went on to generate construct Pti4-7, which contains additional amino acids C-terminal of the ERF (Figure 3A). This construct was found to be expressed in yeast (Figure 3C; lane 8) and was capable of interaction with SEBF (Figure 3B; Pti4-7). Taken together, the data point towards the ERF as the domain interfacing with SEBF. However, we cannot exclude that regions adjacent to the ERF participate in the recruitment by SEBF.

4.3.4 Recruitment of Pti4 to PR-10a Is SEBF-Dependent

Induction of the *PR-10a* gene is positively controlled by Stwhy1, which has been shown, by chromatin immunoprecipitation (ChIP), to be recruited to the gene after wounding or elicitor treatment (Desveaux et al., 2004). The *PR-10a* gene has also been shown to be regulated by repressor SEBF. However, direct binding of the factor to the gene has never been addressed (Boyle and Brisson, 2001). Figure 4A is a diagram of the *PR-10a* gene which shows the position of the PCR primers used for all the ChIP experiments. As reported previously (Desveaux et al., 2004), Figure 4B shows that an enrichment of

Why1 at the *PR-10a* promoter was observed following immunoprecipitations performed with the anti-StWhy1 antibody on wounded (WT-W) and elicited (WT-E) tissues but was absent from the ChIP performed with uninduced (WT-U) samples. Conversely, ChIP performed with the anti-StSEBF antibody led to an enrichment in uninduced tissue, but not in wounded or elicited tissues. This indicates that SEBF is only recruited to *PR-10a* in uninduced conditions, consistent with its role as a transcriptional repressor.

We previously demonstrated, in *Arabidopsis*, that genes negatively regulated by Pti4 and to which Pti4 was shown to be recruited, did not possess a GCC-box, the cognate Pti4 DNA binding element (Chakravarthy et al., 2003). Two models were proposed to explain these observations: first, Pti4 might be recruited directly to these negatively-regulated genes by binding to a novel DNA sequence or second, Pti4 might be indirectly recruited via another DNA-binding protein. Since SEBF (there are 9 *SEBF*-like genes in *Arabidopsis*) is a repressor binding to both *PR-10a* and Pti4, we saw the *PR-10a* gene as an opportunity to test these two models. ChIP performed with anti-StPti4 antibodies showed an enrichment of Pti4 at the *PR-10a* promoter in uninduced tissues but not in wounded or elicited samples (Figure 4B). This demonstrates that Pti4 can be recruited to *PR-10a* despite the absence of a GCC-box and also that its recruitment profile is similar to that of SEBF. The immunoblot of Figure 4C and the qPCR of Figure 4D indicate that both SEBF and Pti4 are present in uninduced, wounded, and elicited tissues and, therefore, that their absence at the *PR-10a* promoter is not due to their absence from the tissue. The data thus suggest that Pti4 might be recruited to *PR-10a* via SEBF. To test this hypothesis, we generated two knock-down lines of SEBF through RNAi technology. The immunoblot of Figure 4E indicates that levels of the SEBF protein

are undetectable in these lines (lanes 2 and 3). ChIP experiments performed with the anti-SEBF antibody on uninduced tissue from the RNAi lines (RNAi#5 and RNAi#14) did not reveal any enrichment of SEBF at the *PR-10a* promoter (Figure 4B). This result indicates that SEBF is not recruited to *PR-10a* in these lines, consistent with the knock-down expression. ChIP experiments performed on the same tissue, but with the anti-Pti4 antibody, also indicated an absence of Pti4 at the *PR-10a* promoter (Figure 4B). The data support the model in which Pti4 is recruited to *PR-10a* via SEBF. To determine the effect of an SEBF knock down on *PR-10a* expression, we performed qPCR on *PR-10a* transcripts in uninduced (U) tissue and after wounding (W) and elicitor (E) treatment (Figure 4F). Silencing of SEBF did not lead to activation of *PR-10a* in uninduced tissues, but both wounding and elicitor treatment led to increased *PR-10a* transcript accumulation in the SEBF RNAi lines when compared to wild-type plants.

4.3.5 The SEBF-Pti4 Complex Forms a Repressosome

The results of Figure 4 demonstrate that Pti4 is recruited to *PR-10a* through interaction with SEBF. However, since Pti4 has been shown to be a transcriptional activator (Gu et al., 2002), we asked ourselves what the molecular consequences of the recruitment of Pti4 to SEBF might be. To address this question, the transcriptional properties of SEBF, Pti4, and the SEBF-Pti4 complex were examined using an in vivo plant transcription assay (Figure 5B). The constructs used in this experiment are detailed in Figure 5A. The baseline level of transcription was determined by transfecting leaves with Gal4 DB (not fused to any other protein or protein domain) along with a reporter construct consisting of a firefly luciferase gene under the control of 5 copies of the Gal4 upstream activating

sequences (UAS) fused to a minimal promoter. Transfection with SEBF:DB resulted in reporter gene activation below the baseline level, consistent with the fact that SEBF is a repressor (Boyle and Brisson, 2001). Co-expression of SEBF:DB and Pti4:TA led to the activation of the reporter gene beyond baseline confirming that the two proteins interact with one another in this plant system. We next addressed how Pti4 would modulate the transcriptional properties of SEBF. SEBF:DB was thus co-expressed with Pti4 (not fused to any foreign transcription activation or DNA-binding domains), which resulted in activation of the reporter gene below baseline. Values were in fact not significantly different from those observed with SEBF:DB, indicating that the SEBF-Pti4 complex, like SEBF, acts as a repressor. To strengthen the argument, we tested the reciprocal constructs. First, however, Pti4:DB was transfected alone and reporter gene activation was monitored. Values were beyond baseline, confirming that Pti4 is a transcriptional activator. Conversely, expression of Pti4:DB along with SEBF (not fused to other protein domains) abolished the capacity of Pti4 to act as a transcriptional activator, as deduced by reporter gene activity falling below baseline. As an additional control, Pti4:DB was further activated by a direct fusion to the VP16TA (Pti4:DB:TA) which led to higher values when compared to Pti4:DB. Reporter gene expression mediated by this construct could also be mitigated by the addition of SEBF (Pti4:DB:TA + SEBF). However, the synergistic effect of the endogenous Pti4 transactivation domain and that of VP16 could not be fully countered by SEBF. Nevertheless, the results of Figure 5 demonstrate that when complexed with SEBF, Pti4 is no longer a transcriptional activator, that is to say, the SEBF-Pti4 complex acts as a repressosome.

4.3.6 Recruitment of SEBF to PR-10a Is Pti4-Dependent

Although results in Figure 5 indicate that the SEBF-Pti4 complex is a repressosome, one question remains unanswered: What is the role of Pti4 in the SEBF-Pti4 complex since SEBF is itself a repressor? To tackle this question, we wanted to test whether recruitment of SEBF to *PR-10a* would be in any way affected by the absence of Pti4. We thus generated two knock-down Pti4 plants. For these experiments, we used virus-induced gene silencing (VIGS) technology, since we failed to recover Pti4 knock-down lines generated by RNAi. Because Pti4 protein levels in wild-type plants are below detection levels when monitored by immunoblot analysis, we assessed the extent of knock-down by qPCR. Figure 6A indicates that levels of the Pti4 mRNA are substantially reduced in Pti4 VIGSed lines (VIGS#1-#4), when compared to the empty vector VIGSed control (PVX-00#1-#4). ChIP experiments performed with the anti-Pti4 antibody on uninduced tissue from the Pti4-VIGSed lines #1 and #2 did not reveal any enrichment (Figure 6B), confirming the absence of Pti4 protein recruitment to *PR-10a* and consistent with the knock-down expression. Interestingly, ChIP experiments performed on the same tissue, but with the anti-SEBF antibody, also indicated an absence of enrichment, which demonstrates that SEBF requires Pti4 for its recruitment to *PR-10a*. The presence of SEBF in these plants was confirmed by immunoblot analysis (Figure 6C) and indicates that the absence of SEBF at the *PR-10a* promoter in Pti4 knocked-down lines is not due to the absence of SEBF in these tissues. As expected, ChIP from empty vector-VIGSed plants (PVX-00#1) revealed the presence of both SEBF and Pti4 at the *PR-10a* promoter after immunoprecipitation with both anti-SEBF and anti-Pti4 antibodies, respectively (Figure 6B). The effect of Pti4 knock-down on *PR-10a* expression was also analyzed by

qPCR. In contrast to SEBF knock-down lines, activation of *PR-10a* was already observed in the absence of any treatment (Figure 6D).

4.4 DISCUSSION

This study was motivated by our interest in elucidating the protein components of the SEBF repressosome. Our data have demonstrated that SEBF is recruited to *PR-10a* under uninducive conditions, but after the gene is activated by wounding or elicitor treatment, the environment at the promoter is not permissive for an interaction with the repressor. Furthermore, our results established that, despite the absence of a cognate GCC-box, Pti4 was recruited to *PR-10a* with a pattern similar to that of SEBF, more specifically, that its interaction occurred only under uninduced conditions. These outcomes, along with the fact that Pti4 was recovered from a yeast two-hybrid screen using SEBF as bait, suggested that Pti4 is drafted to *PR-10a* through recruitment by SEBF. This was confirmed by experiments demonstrating the absence of Pti4 recruitment to *PR-10a* in SEBF knock-down plants. In vivo plant transcription assays demonstrated that the SEBF-Pti4 complex, like SEBF, repressed transcription, indicating that the complex behaves as a repressosome. Finally, Pti4 knock-down lines highlight the raison d'être for the presence of this protein in the repressosome, which is to allow SEBF to reach its DNA target. Together, our data argue that in uninduced conditions SEBF and Pti4 are components of a repressosome that assembles at the *PR-10a* promoter.

4.4.1 SEBF Recruits Pti4 to PR-10a and not Vice Versa

Since ChIP experiments performed on Pti4 knock-down plants demonstrated that Pti4 is also required for the recruitment of SEBF to *PR-10a*, one could argue that Pti4 binds directly to DNA through a previously uncharacterized DNA sequence and that SEBF is drafted to the promoter via Pti4. Although this is a plausible scenario, previous data would argue against this model. Characterization of *PR-10a* through promoter deletion analysis revealed the presence of a negative regulatory element between position -52 and -27 (Després et al., 1995). This identification was followed up by the biochemical purification of the factor binding to this negative element, which was coined SEBF (Boyle and Brisson, 2001). In vitro mutational analysis of the SEBF binding element by EMSA, using recombinant SEBF, allowed us to uncover mutations that would disrupt SEBF DNA-binding activity, but also others that would enhance it. Transcriptional analysis, in potato protoplasts, of modified *PR-10a* promoter variants bearing these mutations highlighted an inverse correlation between transcriptional activity and the recruitment capacity of SEBF to the promoter. DNA mutations that reduced the recruitment of the repressor led to higher reporter gene activity, while those that favored drafting of SEBF reduced it, when compared to a wild-type promoter element or to a mutated variant that did not affect SEBF binding (Boyle and Brisson, 2001). Although Pti4 might be present in potato protoplasts and potentially assisting the binding of SEBF to DNA, EMSA analyses using recombinant SEBF alone, indicate that SEBF can directly bind the SE without Pti4. This clearly demonstrates that repression at the *PR-10a* gene is dependent on direct recruitment of SEBF to its cognate element and indicates that SEBF is indeed the DNA-binding component of the SEBF-Pti4 repressosome. Although we

cannot rule out the possibility that Pti4 could also be associated with a distal GCC-box and interact with SEBF via DNA looping, the fact that it interfaces SEBF via the ERF domain, which coincides with its DNA-binding domain, would suggest that Pti4 may not be able to bind to DNA concomitantly with an interaction with SEBF.

4.4.2 Potential Pti4-Assisted Recruitment Mechanisms of SEBF to PR-10a

ChIP experiments of Figure 6 performed on Pti4 knock-down plants clearly identify Pti4 as mandatory to the in vivo recruitment of SEBF to *PR-10a*. In vitro, on the other hand, SEBF can readily interact with its cognate DNA element (Figure 2E; Boyle and Brisson, 2001). However, in vitro, SEBF only recognizes one strand of the SE, the coding strand, but cannot bind the non-coding strand or the double stranded DNA (Boyle and Brisson, 2001). Thus a handful of scenarios may provide a rationale for the role of Pti4 in allowing SEBF recruitment to *PR-10a* and these depend on the architecture of the SE in uninduced conditions.

First, the SE could be in a single-stranded DNA conformation. Under this condition one would assume, however, that SEBF could bind directly to its cognate ssDNA and a requirement for Pti4 does not appear obvious. Nevertheless, in this scenario, Pti4 could prevent dismissal of SEBF from *PR-10a* by precluding, for example, the occurrence of post-translational modifications or other SEBF-protein interactions that could result in decreased DNA-binding affinity. The opposite setting, in which Pti4 would favor the recruitment of additional co-factors stimulating the post-translational modification of SEBF and allowing its stronger interaction with DNA, is as probable.

Second, the SE could be in a double-stranded DNA conformation in vivo, under uninduced conditions, and prior to binding of SEBF or the SEBF-Pti4 complex. Although this seems unlikely given that SEBF cannot bind dsDNA in vitro, this behavior parallels the activity of some heterogeneous nuclear ribonucleoproteins (hnRNP). hnRNPs are among the best characterized ssDNA binding proteins involved in transcriptional regulation. They can activate or repress gene expression (Hsieh et al., 1998; Ostrowski et al., 2003). Like SEBF, hnRNPs are found in many subcellular (Swanson and Dreyfuss 1988; Ostrowski et al., 1991; 2002) compartments and contain regions similar to the cs-RBD domains (Boyle and Brisson, 2001). In an exemplary case, hnRNP C1/C2 purified from in vivo source with its associated co-factors could bind the double-stranded DNA version of its cognate sequence, while the recombinant version, devoid of co-factors, cannot (Mahajan et al., 2005). The same rationale could be applied to SEBF, where Pti4 may stimulate a latent helicase activity in SEBF or help recruit such an activity to the repressosome as is the case in the hnRNP K-Purα repressosome (Da Silva et al., 2002). Although it is unclear at present which mechanism governs the Pti4-assisted recruitment of SEBF to *PR-10a*, these proposed scenarios constitute future areas of potential exploration.

4.4.3 A Working Model of the PR-10a Gene Regulation

Figure 7 summarizes our results and provides a model for the regulation of *PR-10a* through the interplay of a repressosome and an activator. In the uninduced state, the SEBF-Pti4 repressosome complex occupies the SE while StWhy1 is stored inactive and sequestered away from the ERE (Desveaux et al., 2000). Upon induction, the

repressosome is dismissed from the promoter and the DNA-binding activity of StWhy1 is induced, allowing the ERE to recruit the activator StWhy1 (Desveaux et al., 2000; Desveaux et al., 2004).

We have previously shown that transgenic lines carrying a reporter gene regulated by the first 135 base pairs of the *PR-10a* promoter and lacking the SE is not activated in uninduced tissues, but still requires wounding or elicitor treatment for expression of the transgene (Després et al., 1995). This is consistent with the observation that the expression of *PR-10a* in the SEBF RNAi lines (Figure 4F), where the repressosome is not recruited to the promoter, still requires induction by wounding or elicitor treatment. These observations are also in agreement with current models of gene regulation, where derepression (removal of repressing marks and/or proteins) and activation are viewed as distinct phenomena. In other words, the absence of a repressor at a promoter does not equate to gene activation. Activation requires the recruitment of activators (Roeder, 2005). Since the SEBF-Pti4 repressosome is not recruited to *PR-10a* after wounding or elicitor treatment, the fact that transcript accumulation is higher under these conditions in the RNAi lines, when compared to wild-type plants, cannot be well explained by our simple model. However, gene regulation involves more than just transcription factor recruitment and an important aspect of controlling genes resides in remodeling chromatin architecture (Roeder, 2005). We thus propose that a complete absence of SEBF from the promoter, whether through a knock-down of SEBF, as in our RNAi lines, or by deleting the SE element from a reporter gene (Després et al., 1995), might lead to a more open and permissive chromatin architecture allowing easier access of the activator Why1 to the *PR-10a* promoter after wounding or elicitor treatment.

The observation that *PR-10a* was activated in the uninduced Pti4 VIGSed plants is surprising, as one would have expected results similar to those obtained with the SEBF knock-down lines, since in both cases the repressosome is not recruited to *PR-10a*. A possible explanation is that the Pti4-VIGSed plants are rendered more responsive than the control plants due to priming by the virus used to propagate the silencing construct. This priming might involve the activation of Why1, which could be recruited by *PR-10a* resulting in activation of the gene. In PVX-00 plants, however, the presence of the SEBF-Pti4 repressosome at the *PR-10a* promoter may be sufficient to occlude the active Why1 from gaining access to the ERE. Another explanation could be that knocking-down Pti4 affects not only the repressosome, but also activation pathways, such as those controlling the activation of StWhy1. These are interesting questions that deserve further investigations.

The data presented here provide a significant advancement of our mechanistic understanding of gene regulation at the defense-associated gene *PR-10a* and illustrates how precarious and misleading our attempts at categorizing transcription factors as activators or repressors can be. The data presented here elevate Pti4 to the status of plant paradigm of transcription factor duality, capable of both activating and repressing transcription in a context-dependent fashion.

4.5 METHODS

4.5.1 Plant Material and Chemicals

Solanum tuberosum cv Kennebec plants were grown on soil in a growth chamber at 60% humidity and under a long day photoperiod consisting in a 16-hr light regimen with a photosynthetic photon flux density of 150 μmol photons m^{-2} s^{-1} at 23°C followed by an 8-hr dark period at 18°C. All chemicals were from Sigma-Aldrich, unless otherwise stated.

4.5.2 Yeast Two-Hybrid Screening and Interaction Domains Mapping

The yeast two-hybrid transformation and screening as well as quantitative β-galactosidase activity assays were done according to the protocol of the "DupLEX-A Yeast Two-Hybrid System" (OriGene Technologies, Rockville, MD). The tomato cDNA library used as prey was constructed in the laboratory of Barbara Baker and is described elsewhere (Zhang et al., 1999). The cDNA sequence encoding the mature potato SEBF protein was cloned in the BamHI/XhoI sites of the bait vector pEG202 to produce a C-terminal protein fusion with the Lex-A DNA binding domain. Potato Pti4 was PCR-amplified from a potato cDNA library (Matton and Brisson, 1989) using the oligonucleotides 5'-AAAGCCATATGGATCAACAGTTACCACCGA-3' and 5'-TTCGGCTCGAGAATGACCAATAGTTGATGGA-3', which were based on the tomato Pti4 cDNA sequence. Potato Pti4 was subcloned in the NdeI and XhoI sites of plasmid pET-21a (Novagen, Madison, WI).

For mapping the StSEBF-StPti4 interacting domains, fusions between the Lex-A DNA binding domain of pEG202 and either the full length SEBF or its truncated versions were created by PCR amplification (see Supplemental Table 1 for the sequences of the oligonucleotides used). Fusions of HA-tagged StPti4, or deleted variants of StPti4, with

the transactivation domain B42 of pJG4-5 were also generated by PCR (see Supplemental Table 1 for the sequences of the oligonucleotides used).

4.5.3 Immunoblot

The expression of fusion proteins produced in yeast was confirmed by immunoblot analysis using an anti-LexA antibody (Invitrogen, Burlington, Ontario) for SEBF fusions to the LexA DB and an anti-HA antibody (Santa Cruz, California) for the Pti4 constructs. Analysis of SEBF from WT and SEBF RNAi potato plants was performed using an anti-SEBF antibody (Boyle and Brisson, 2001).

The expression of SEBF and Whirly proteins in leaves of PVX-infected plants and in treated and untreated potato tubers was determined as described (Constabel and Brisson, 1992) but using the anti-SEBF antibody at a 1:5000 dilution and the anti-StWhy1 antibody at a 1:4000 dilution (Desveaux et al., 2000).

4.5.4 Pull Down Assays

Mature StSEBF cDNA was cloned in pET21 (Novagen, Madison, WI) and expressed as a C-terminal His-tag fusion. StSEBF-His protein was immobilized and purified on a Ni-NTA column (Qiagen) following the manufacturer's instructions. Pti4 was cloned into the yeast expression vector pJG4-5 and expressed as a B42-HA-tag fusion. Yeast protein extracts expressing Pti4-B42-HA or B42-HA alone, which served as a negative control, were loaded on to an empty Ni-NTA column (another negative control) or to one bound by SEBF-His. The columns were washed three times in 50 mM NaH_2PO_4, 300 mM NaCl,

20 mM imidazole, pH 8.0 before eluting in the same buffer containing 500 mM imidazole. Proteins were then separated on a 12% SDS–polyacrylamide gel and transferred to a membrane for immunoblot analysis. Anti-HA (Santa Cruz, California) or anti-SEBF (Boyle and Brisson, 2001) antibodies were used to detect Pti4-HA or SEBF-His fusion proteins, respectively.

4.5.5 Electrophoretic Mobility Shift Assays (EMSA)

EMSAs were carried out with recombinant full length and truncated SEBF proteins, as previously described by Boyle and Brisson (2001), using as a probe the oligonucleotide SE PR-10a (5'-TCTAGACTGTCACTTGTTTTT-3').

4.5.6 Stable Transformation of Potato with a Hairpin-Driven RNAi Construct

A 700 pb fragment from the cDNA of potato SEBF was amplified by PCR using the primers SEBF-BamHI (5'-TTTGTTCGGATCCTAACGCTTTC-3') and SEBF-KpnI (5'-GTTGGGTACCATCTTCAGAATTG-3') to generate the sense construct and primers SEBF-ScaI (5'-GGCTAAGTACTTCAGAATTGACGTC-3') and SEBF-SacI (5'-GTTTTGAGCTCAAAGTAACCCTTTC-3') for the antisense one. The sense and antisense PCR products were subcloned in the pDarth vector (O'Brien et al., 2002) using restriction sites *Bam*HI/*Kpn*I and *Sca*I/*Sac*I, respectively. Transformation in Agrobacterium and in potato plants was as described (Després et al., 1995).

4.5.7 Chromatin Immunoprecipitation (ChIP)

Two grams of tissue per experiments were treated and processed for ChIP analysis as previously described (Desveaux et al., 2004 and Chakravarthy et al., 2003). The antibodies used for the ChIPs were anti-StWhy1 (Desveaux et al., 2000), anti-SEBF (Boyle and Brisson, 2001), and anti-Pti4 (Chakravarthy et al., 2003). The sequences of the oligonucleotides used to amplify the *PR-10a* promoter are: 5'-AAGAAGGCACATTTCAAGAAC-3' and 5'-ACCTATAAATACCATCGAACA-3'. Biological replicates of ChIP were performed from each genotype/treatment sample and three qPCR experiments were done with each sample. The qPCR reactions were performed with 40 cycles of a two-temperature protocol in a total volume of 20 µl using the Bio-Rad (Mississauga, ON) iQ SYBR Green Supermix kit according to the manufacturer's instructions. To amplify the *PR-10a* promoter with the oligonucleotides described above, an annealing temperature of 55°C was used. The sequences of the oligonucleotides used to amplify the *Actin* promoter were: 5'-ACTATTATTCAATTTATCTGCGGCC-3' and 5'- AAAAATGGCAGGCCAACTCT -3' and an annealing temperature of 64°C was used. For each immunoprecipitation (IP), binding of a transcription factor (SEBF, Why1, or Pti4) to the *PR-10a* promoter relative to its binding at the *Actin* promoter was determined with the following formula: amount *PR-10a* promoter (IP)/amount *Actin* promoter (IP). The amount of target DNA was defined as 2^{-Ct}, where Ct is the threshold cycle.

4.5.8 In vivo Plant Transcription Assays

The construction of the reporter gene and the internal standard vectors as well as the methodology for the in vivo plant transcription assays were previously described (Després et al., 2003). N-terminal protein fusions of Pti4 and SEBF with the Gal4 DB and VP16 TA and the unfused versions were created by PCR amplification and cloned into pBI524 as previously described (Després et al., 2003).

4.5.9 Virus Induced Gene Silencing in Solanum tuberosum cv Kennebec

The protocol for virus induced gene silencing of *S. tuberosum* cv Kennebec was based on that previously described by Faivre-Rampant and collaborators (2004) with some modifications. The PVX vector pGR106 (Lu et al., 2003) was obtained from David Baulcombe (Sainsbury Laboratory, Norwich, UK). To construct the PVX-Pti4, a 335 bp PCR fragment was amplified from *S. tuberosum* cv Kennebec cDNA using the oligonucleotides StPti4NotI-F (5'-AGCGGCCGCGAAACACCGAAGGGAAGACA-3') and StPti4AscI-R (5'-AGGCGCGCCCTCCACTCCTCCGTCACATT-3') and subcloned into the NotI/AscI restriction sites of pGR106. Each of the PVX construct (PVX-Pti4 and the empty vector PVX-00) were co-transformed with the helper plasmid pSoup (Hellens et al., 2000) into *Agrobacterium tumefaciens* strain LB4404 by electroporation.

Potato plants were propagated in vitro as described (Faivre-Rampant *et al,* 2004), but with some modifications. Five stem pieces per Magenta box were cultivated in 100 mL of MS medium (Murashige and Skoog, 1962) supplemented with 0.4 mg/mL thiamine, 0.5 mg/mL pyridoxine, 0.5 mg/mL nicotinic acid, 100 mg/mL myo-inositol, 2

mg/mL glycine, 30 g/L sucrose, 1 mg/L IBA, 0.2 mg/L kinetin, 12 mM $AgNO_3$, and 96 mM $Na_2S_2O_3$.

Ten days to two weeks old in vitro plants were agroinoculated with the PVX vectors. The different Agrobacterium strains were grown for two days at 28°C with shaking and then incubated in an induction buffer (10 mM MES, 10 mM $MgCl_2$, and 200 μM acetosyringone) for at least 3 hours at room temperature. After induction, the bacterial suspension was pelleted and used to inoculate the surface of leaves, which were previously wounded with a razor blade to facilitate bacterial penetration. Two weeks later, the plants were transferred to soil in controlled-environment chambers with a 16 hr-photoperiod. Five to 8 weeks later, the plants were analyzed by real time PCR for *StPti4* levels as indicated bellow. Plants demonstrating no or highly reduced Pti4 transcript levels were chosen for ChIP experiments and *PR-10a* expression analysis. Four biological replicates were used for qPCR experiments.

4.5.10 RNA Extractions and Real-Time Quantitative PCR (qPCR)

RNA from four biological replicates of potato leaves was extracted using the Tri-Reagent method (Sigma-Aldrich) following the manufacturer's instructions specific for high polysaccharide-containing samples. First-strand cDNA was synthesized from 2 μg of total RNA using Superscript II reverse transcriptase (Invitrogen, Burlington, Ontario) and a polyT oligonucleotide, following the manufacturer's instructions. For each biological replicate a pool of five leaves was used.

RNA from three biological replicates of potato tubers was extracted as previously described (Boyle et al., 2001). For each biological replicate a pool of three tuber disks

was used. qPCR was performed using the SYBR® green method. SYBR® green PCR reactions were performed using 2 µl of cDNA samples (50 ng), 5 µl of the Fast SYBR® Green Master Mix (Applied Biosystem, CA), and 10 pmol of each primer in a total volume of 10 µl. Melting curves were performed using the dissociation curve software SDS 2.2.2 to ensure that only a single product was amplified. For quantification of *Pti4* transcript levels in VIGSed plants, a forward primer (St-Pti4qPCR-F1 5'-TCACCGCCGGCGAAGTAAA-3') located outside of the sequence targeted for silencing and a reverse primer (StPti4qPCR-R1 5'-CGTTAGACAGCGGCCGTGG-3') located inside the sequence targeted for silencing were used. *PR10-a* quantification was carried out using the primers PR10a-F 5'- TGACAATCTTATTCCTAAGTTGATGC-3' and PR10a-R 5'- AGGTCATCTTCTTGATGCTTCC-3'. As an endogenous control, the primers Ubiq-F 5'-CTCCGTGGTGGTATGCAGAT-3' and Ubiq-R 5'-CACGTTGTCAATGGTGTCG-3' were designed for quantification of the ubiquitin gene (accession number BQ045862). The ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems, CA) was used to detect the amplification level and was programmed with an initial step of 10 minutes at 95°C, followed by 45 cycles alternating between 15 sec at 95°C and 1 min at 60°C. All reactions were run in technical triplicate for each biological replicate and the average values were used for quantification. The relative quantification of target genes was determined by using the △△CT method. Briefly, the C_t (threshold cycle) values of target genes were normalized to an endogenous control gene (ubiquitin) ($\triangle CT = Ct_{target} - Ct_{Ubiquitin}$) and compared with a calibrator: $\triangle\triangle CT = \triangle Ct_{Sample} - \triangle Ct_{Calibrator}$. Relative expression (RQ) was calculated using the

Sequence Detection System SDS 2.2.2 software (Applied Biosystems) and the formula $RQ = 2^{-\Delta\Delta CT}$.

4.5.11 Accession Numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers AF389431 (StSEBF), U89255 (SlPti4), EU851735 (StPti4) and X55751 (*S. tuberosum* actin gene PoAc97).

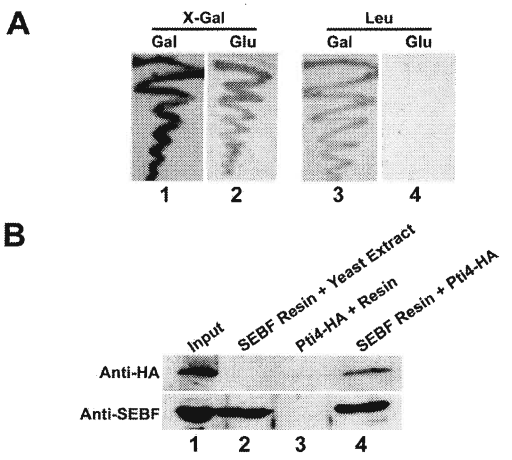


Figure 1. The Repressor SEBF Interacts with the Transcriptional Activator Pti4.

(A) The yeast two-hybrid interaction between the SEBF bait and the Pti4 prey was revealed through the expression of the *lacZ* reporter gene (β-galactosidase activity), detected as a blue color (shown here in dark-grey/black) in plates containing galactose (Gal) and supplemented with X-Gal (lane 1), or by prototrophic growth (activation of the *LEU2* reporter gene) in medium lacking leucine (Leu), but containing galactose (lane 3). The activation of the reporter genes was dependent on the expression of the prey construct, since its suppression in medium containing glucose (Glu) does not lead to the activation of the *lacZ* reporter gene (lane 2) or to prototrophic growth (lane 4).
(B) The pull down assays were performed by incubating SEBF produced in *E. coli* and coupled to a solid support with Pti4 expressed as an HA-fusion protein in yeast (lane 4). As negative controls, pull-downs were performed by omitting SEBF (lane 3) or Pti4 (lane 2). Lane 1 contains 100% of the amount of SEBF coupled to the solid support (lower panel) or 20% of the amount of Pti4 used in the pull-down experiments (upper panel). Proteins were analyzed by immunoblotting using an anti-HA antibody for Pti4 detection (upper panel) or an anti-SEBF antibody (lower panel).

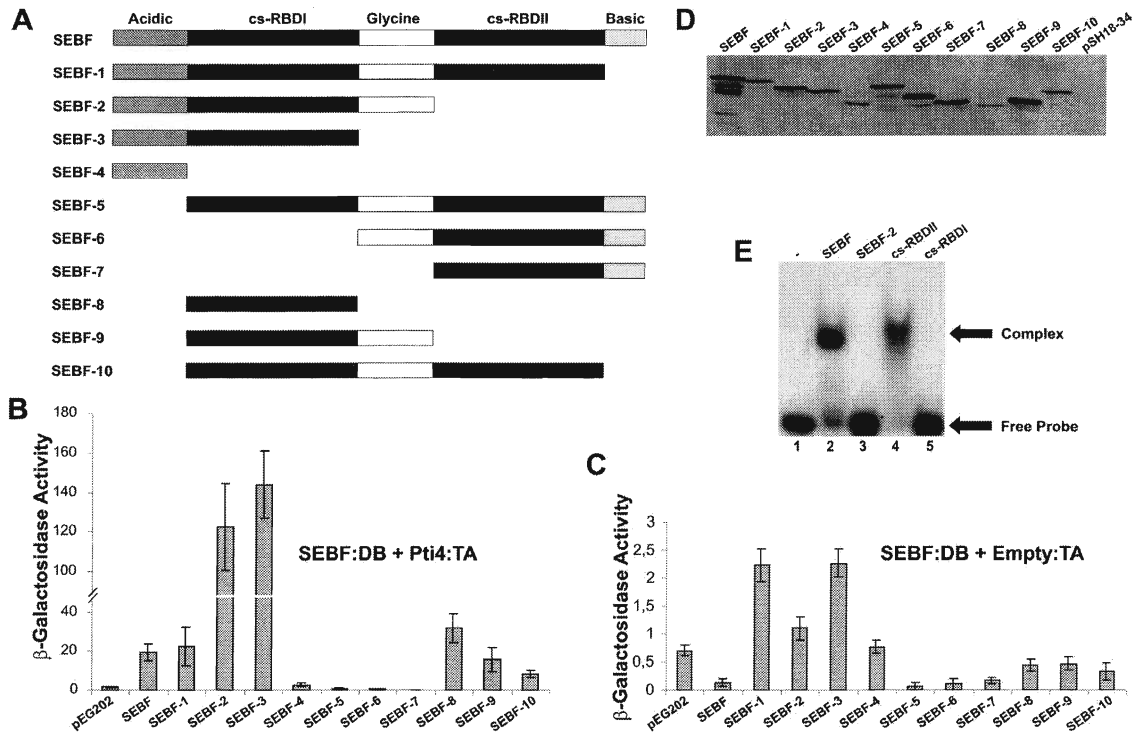


Figure 2. SEBF Interacts with DNA through cs-RBDII and Recruits Pti4 via cs-RBDI.

(A) Schematics of SEBF and its deletion mutants analyzed in panels **(B)** to **(E)**. "Acidic" and "basic" indicate domains with these properties, while "glycine" represents a glycine-rich region of SEBF. "cs-RBDI" and "cs-RBDII" refer to consensus sequence-type RNA-binding domains I and II.
(B) Bar diagram illustrating the interaction of SEBF and the mutants depicted in **(A)** fused to the Lex-A DB with the full length Pti4 fused to the B42 TA.
(C) Bar diagram illustrating the background level of activity observed with each of the SEBF constructs depicted in **(A)** and co-expressed with the empty B42 TA vector. For **(B)** and **(C)**: Results obtained by expressing the Lex-A DB alone (pEG202) along with Pti4:TA are shown as a reference baseline value. Note the difference in scales between these panels. Values consist of n=6 samples and represent averages ± 1 SD. Every bar represents an assay on 3 different colonies repeated on two independent transformation events.
(D). An immunoblot using an anti-SEBF antibody was performed to confirm the expression of SEBF and its mutant derivatives in cell lines used in **(B)**.
(E) EMSA analyses were carried out with the full length SEBF (lane 2), SEBF-2 (lane 3), the cs-RBDI domain of SEBF (SEBF-8 in **(A)**; lane 4), and the cs-RBDII domain of SEBF (lane 5). All the studies were done with 10 ng of purified recombinant protein and the ^{32}P-labeled single-stranded SE oligonucleotide. Lane 1 contained only the labeled SE oligonucleotide.

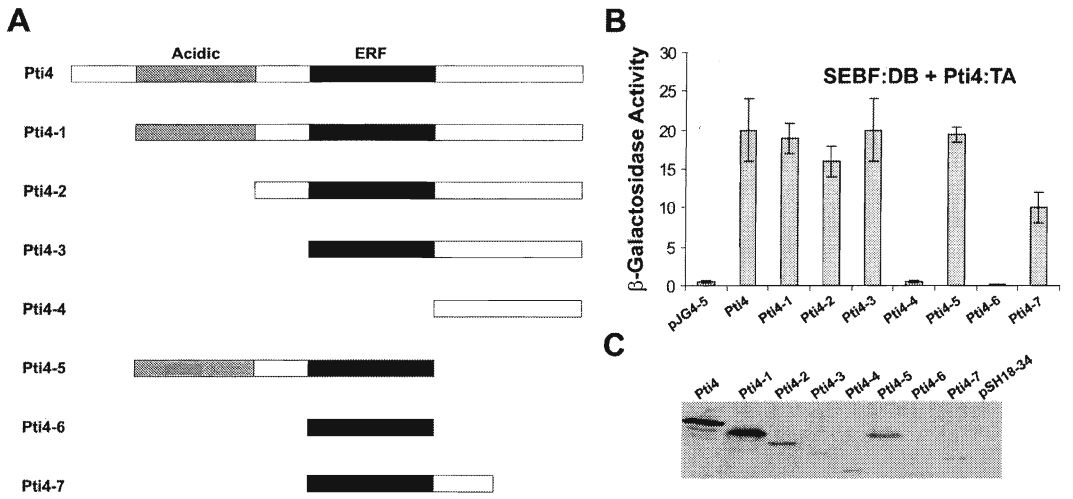


Figure 3. The ERF Domain of Pti4 Interfaces with SEBF.

(A) Schematics of Pti4 and its deletion mutants analyzed in panels **(B)** to **(D)**. "Acidic" indicates a domain with this property, while "ERF" stands for the ethylene-response factor domain, which contains the DNA-binding region of Pti4.
(B) Bar diagram illustrating the interaction of Pti4 and the mutants depicted in **(A)** fused to the B42 TA containing an HA-Tag along with the full length SEBF fused to the Lex-A DB. Results obtained by expressing the B42 TA alone (pJG4-5) along with SEBF:DB are shown as a reference baseline value. Note the difference in scales between these panels. Values consist of n=6 samples and represent averages ± 1 SD. Every bar represents an assay on 3 different colonies repeated on two independent transformation events.
(C) An immunoblot using an anti-HA antibody was performed to confirm the expression of Pti4 and its mutant derivatives in cell lines used in **(B)**.

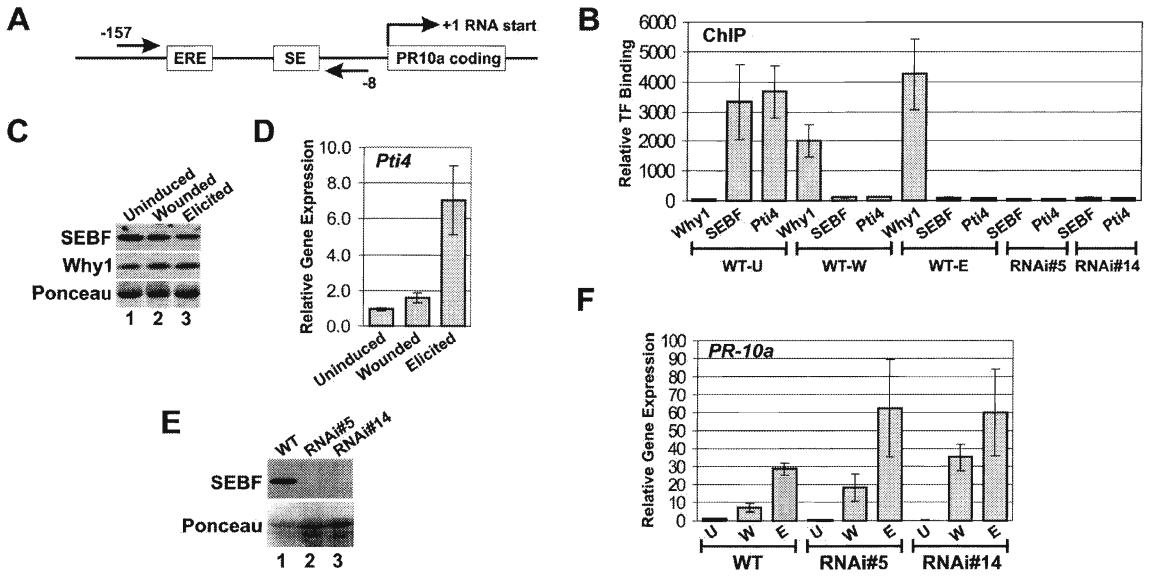


Figure 4. Pti4 Binds to the *PR-10a* Promoter through its Interaction with SEBF.

(A) Diagram of the *PR-10a* promoter showing the position of the elicitor response element (ERE), the silencing element (SE) and the oligonucleotides used for the ChIP experiments. The straight arrows and numbers refer to the location of the oligonucleotides with respect to the RNA start site.
(B) ChIP experiments analyzed by qPCR. WT-U, WT-W, and WT-E indicate ChIPs performed in wild-type plants that were left uninduced, or were wounded, or treated with the elicitor, respectively. ChIPs were also conducted with uninduced tissues from two independent SEBF RNAi lines (RNAi#5 and RNAi#14). ChIPs were performed with an anti-Why1 antibody (Why1), an anti-SEBF antibody (SEBF), or an anti-Pti4 antibody (Pti4). The amount of the different transcription factors binding to *PR-10a* was relative to their recruitment to the *Actin* gene PoAc97 (see Methods). Data for each bar are from 3 biological replicates and errors are equal to ± 1 SD.
(C) The two top panels are immunoblot analysis of SEBF and Why1 proteins extracted from uninduced, wounded, or elicited wild-type plants. An anti-SEBF or anti-StWhy1 antibody was used. The bottom panel is a Ponceau staining of the membrane, shown in the top panels, as a loading control. One representative replicate out of three is shown.
(D) Bar diagram illustrating the abundance of *Pti4* transcript in uninduced, wounded, or elicited wild-type plants. Values represent mean ± 1 SD from three biological replicates.
(E) The top panel is an immunoblot analysis of SEBF proteins extracted from wild-type plants (WT), SEBF RNAi line 5 (RNAi#5), and SEBF RNAi line 14 (RNAi#14). An anti-SEBF antibody was used. The bottom panel is a Ponceau staining of the membrane, shown in the top panel, as a loading control. One representative replicate out of three is shown.
(F) Bar diagram illustrating the abundance of *PR-10a* transcript in uninduced (U), wounded (W), or elicited (E) plants from wild-type (WT) plants, or from two SEBF RNAi lines (RNAi#5 and RNAi#14). Values represent mean ± 1 SD from three biological replicates.

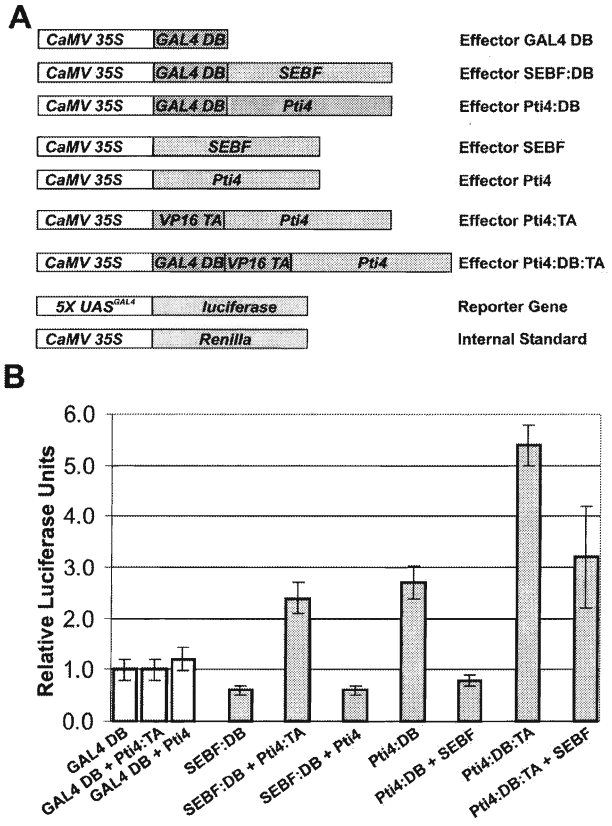


Figure 5. The SEBF-Pti4 Complex Represses Transcription In Vivo.

(A) Representation of the different constructs used in the plant two-hybrid and in vivo transcription assays. Promoters are shown in white boxes. *CaMV 35S* indicates the double *Cauliflower mosaic virus 35S:Alfalfa mosaic virus* promoter. *5X UASGAL4* indicates a promoter composed of a multimerized (five elements) Gal4 upstream activating sequence fused to a minimal TATA box and the Ω translational enhancer from the *Tobacco mosaic virus*. Coding sequences are shown in dark and light gray boxes. *GAL4 DB* indicates the *GAL4* DNA binding domain. *VP16 TA* indicates the constitutive transactivation domain of viral protein 16. All constructs possess the polyadenylation signal from the *nopaline synthase* gene (not shown). The 35S:Renilla construct is an internal reference to normalize transfection efficiency.
(B) Bar diagram illustrating the interaction of Pti4 with SEBF as well as the transcriptional activation potential of Pti4, SEBF, and the SEBF-Pti4 complex. Each effector or pair of effectors was co-transfected with the reporter gene and the internal standard. The effector construct containing the *GAL4 DB* only was transfected into untreated leaves along with the reporter and internal standard constructs and was given an arbitrary value of 1 relative luciferase unit ± 1 SD after normalization with Renilla activity. All values are relative to the activity of Gal4 DB obtained in untreated leaves. Values consist of n=25 samples and represent averages ± 1 SD. Every bar represents five bombardments repeated five times (n=25). White bars represent Gal4 DB controls, while grey bars refer to data obtained with the proteins under investigation.

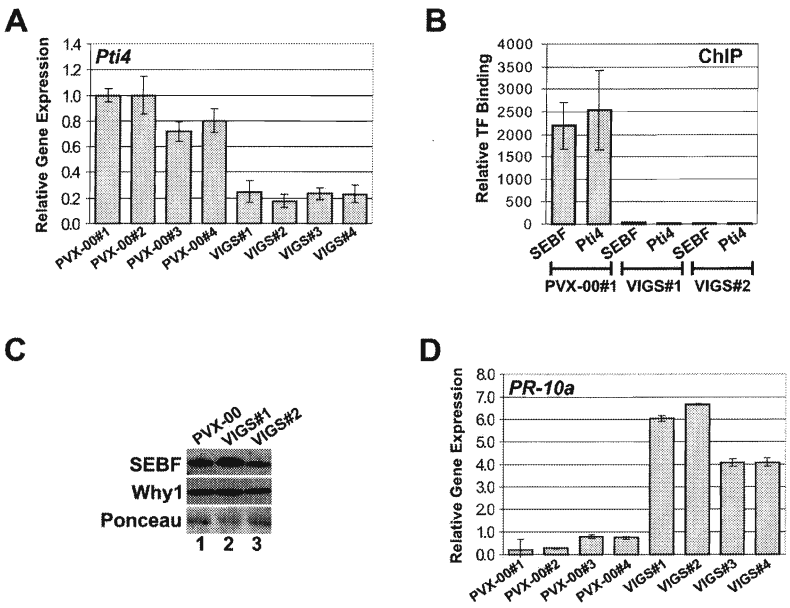


Figure 6. Pti4 is Required for Binding of SEBF to the *PR-10a* Promoter.

(A) Bar diagram illustrating the abundance of *Pti4* transcript in PVX-Pti4 VIGS lines (VIGS#1-4) relative to PVX-00 lines (PVX-00#1-4). Data for each of the 4 biological replicates (each bar) are averages of 3 technical replicates and errors are equal to ± 1 SD.
(B) ChIP experiment analyzed by qPCR. The amount of the different transcription factors binding to *PR-10a* was relative to their recruitment to the *Actin* gene PoAc97 (see Methods). ChIPs from two independent PVX-Pti4 VIGS lines (VIGS#1 and VIGS#2) and from a VIGS line containing the empty pGR106 vector (PVX-00) were performed with uninduced tissues. ChIPs were conducted with an anti-SEBF (SEBF) or an anti-Pti4 (Pti4) antibody. Data for each bar are from 2 biological replicates and errors are equal to ± 1 SD.
(C) The two top panels are immunoblot analysis of SEBF and Why1 proteins extracted from the PVX-00 line, and from the PVX-Pti4 VIGS lines #1 and #2 (VIGS#1 and VIGS#2). An anti-SEBF or anti-StWhy1 antibody was used. The bottom panel is a Ponceau staining of the membrane, shown in the top panels, as a loading control.
(D) Bar diagram illustrating the abundance of *PR-10a* transcript present in the PVX-00 lines (PVX-00#1-4), and in the PVX-Pti4 VIGS lines (VIGS#1-4). Data for each of the 4 biological replicates (each bar) are averages of 3 technical replicates and errors are equal to ± 1 SD.

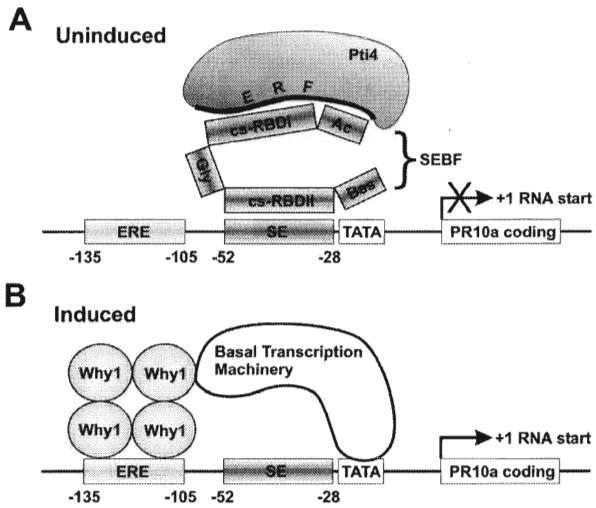
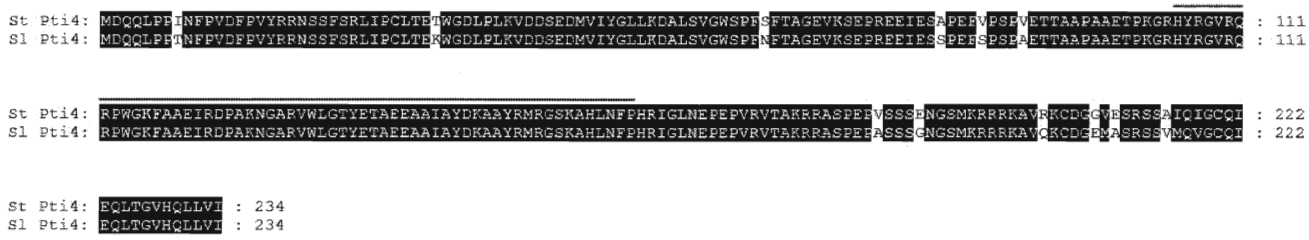


Figure 7. Model for the Transcriptional Regulation of *PR-10a* by the Transcription Factors StWhy1, SEBF, and Pti4.

(A) In uninduced tissues, the repressosome SEBF-Pti4 is recruited to the SE (silencer element) through the cs-RBDII (consensus sequence-type RNA-binding domain II) of SEBF, while the csRBDI and potentially the acidic domain (Ac) of SEBF are required for interfacing with the ERF (ethylene-response factor) domain of Pti4. Recruitment of the repressosome to *PR-10a* prevents transcription through a yet to be identified mechanism. "Bas" and "Gly" indicate a basic domain and a glycine-rich region, respectively. Their functions are unknown. "TATA" refers to the TATA-box.
(B) In induced tissues, the SEBF-Pti4 repressosome is dismissed from the *PR-10a* promoter, while the ERE (elicitor-response element) is populated by the transcriptional activator StWhy1, which is a mandatory step in the transcriptional activation of *PR-10a*. According to the accepted gene activation paradigm, StWhy1 would contact, directly or indirectly, the basal transcription machinery. In **(A)** and **(B)**, the numbers indicate the nucleotide position in the promoter with respect to the RNA start site.



Supplemental Figure 1. Protein Sequence Alignment between Tomato and Potato Pti4.

Protein sequence comparison between potato St Pti4 and tomato Sl Pti4 was performed using CLUSTALW software (Thompson et al., 1994) and edited using the GeneDoc program. Black shading indicates that a residue is conserved in both sequences. The bar above the sequences indicates the position of the ERF motif.

Nicolas, K.B., and Nicolas, H.B. GeneDoc: a tool for editing and annotating multiple sequence alignment; http://www.psc.edu/biomed/genedoc.

		Exp 1	Exp 2	Exp 3	Average 2EΔCt	SD 2EΔCt
WT-U Why1	PR10 Ct	31.37804	29.33421	30.24562		
	Actin Ct	35.25327	33.77821	34.89632		
	ΔCt	3.87523	4.444	4.6507		
	2EΔCt	14.67	21.77	25.11	**20.51666667**	**5.331653902**
WT-U SEBF	PR10 Ct	21.64085	22.55351	22.40737		
	Actin Ct	32.89126	33.98562	35.01739		
	ΔCt	11.25041	11.43211	12.21002		
	2EΔCt	2436.19	2763.17	4737.86	**3312.406667**	**1245.257758**
WT-U Pti4	PR10 Ct	20.93902	19.62159	23.42584		
	Actin Ct	32.87523	31.71806	34.82645		
	ΔCt	11.93621	12.09647	11.40061		
	2EΔCt	3918.84	4379.26	2703.5	**3667.2**	**865.7568432**
WT-W Why1	PR10 Ct	24.85135	23.68054	23.94752		
	Actin Ct	35.37766	35.00201	34.88123		
	ΔCt	10.52631	11.32147	10.93371		
	2EΔCt	1474.81	2559.12	1956.03	**1996.653333**	**543.2952562**
WT-W SEBF	PR10 Ct	24.11711	29.48868	27.96593		
	Actin Ct	30.89002	35.99157	34.6655		
	ΔCt	6.77291	6.50289	6.69957		
	2EΔCt	109.36	90.69	103.94	**101.33**	**9.604754031**
WT-W Pti4	PR10 Ct	26.55533	22.76632	28.28434		
	Actin Ct	33.4583	29.76921	34.98715		
	ΔCt	6.90297	7.00289	6.70281		
	2EΔCt	119.67	128.26	104.17	**117.3666667**	**12.20905538**
WT-E Why1	PR10 Ct	17.16795	24.05903	22.54951		
	Actin Ct	28.96348	35.85911	35.00561		
	ΔCt	11.79653	11.80008	12.4561		
	2EΔCt	3557.21	3565.97	5619.01	**4247.396667**	**1187.860066**
WT-E SEBF	PR10 Ct	23.66904	20.61982	27.91585		
	Actin Ct	30.15827	27.02021	34.6248		
	ΔCt	6.48923	6.40039	6.70895		
	2EΔCt	89.84	84.47	104.62	**92.97666667**	**10.43477998**
WT-E Pti4	PR10 Ct	28.50038	28.10748	27.93515		
	Actin Ct	34.48261	34.00731	33.95047		
	ΔCt	5.98223	5.89983	6.01532		
	2EΔCt	63.22	59.71	64.68	**62.53666667**	**2.554492774**
#5-U SEBF	PR10 Ct	24.18392	23.44011	24.22214		
	Actin Ct	28.45063	28.178	29.11998		
	ΔCt	4.26671	4.73789	4.89784		
	2EΔCt	19.25	26.68	29.81	**25.24666667**	**5.423949975**
#5-U Pti4	PR10 Ct	23.88816	29.83268	29.31684		
	Actin Ct	28.9902	35.23365	35.02943		
	ΔCt	5.10204	5.40097	5.71259		
	2EΔCt	34.35	42.25	52.44	**43.01333333**	**9.069125279**
#14-U SEBF	PR10 Ct	25.14659	28.36627	23.59425		
	Actin Ct	31.67782	35.05601	29.90337		
	ΔCt	6.53123	6.68974	6.30912		
	2EΔCt	92.49	103.23	79.29	**91.67**	**11.99104666**
#14-U Pti4	PR10 Ct	27.06047	27.3171	22.66439		
	Actin Ct	32.55657	33.38233	29.00446		
	ΔCt	5.4961	6.06523	6.34007		
	2EΔCt	45.13	66.96	81.01	**64.36666667**	**18.08003411**

Supplemental Figure 2. Data from the ChIP Experiments of Figure 4B.

		Exp 1	Exp 2	Average 2EΔCt	SD 2EΔCt
PVX-00#1	PR10 Ct	16.55307	20.24065		
SEBF	Actin Ct	27.38911	31.56322		
	ΔCt	10.83604	11.32257		
	2EΔCt	1827.99	2561.14	**2194.565**	**518.4153366**
PVX-00#1	PR10 Ct	17.468	19.81824		
Pti4	Actin Ct	28.37006	31.44671		
	ΔCt	10.90206	11.62847		
	2EΔCt	1913.58	3166.05	**2539.815**	**885.6300302**
VIGS#1	PR10 Ct	28.6722	25.85549		
SEBF	Actin Ct	33.99715	30.68479		
	ΔCt	5.32495	4.82934		
	2EΔCt	40.08	28.43	**28.43**	**8.237794001**
VIGS#1	PR10 Ct	24.64282	22.32973		
Pti4	Actin Ct	29.11485	27.03207		
	ΔCt	4.47203	4.70234		
	2EΔCt	22.19	26.03	**24.11**	**2.71529004**
VIGS#2	PR10 Ct	28.14033	29.17724		
SEBF	Actin Ct	32.44661	32.97134		
	ΔCt	4.30628	3.7941		
	2EΔCt	19.78	13.87	**16.825**	**4.179001077**
VIGS#2	PR10 Ct	27.30008	25.2324		
Pti4	Actin Ct	30.99789	28.15387		
	ΔCt	3.69781	2.92147		
	2EΔCt	12.98	7.58	**10.28**	**3.818376618**

Supplemental Figure 3. Data from the ChIP Experiments of Figure 6B.

Oligonucleotide name	Sequence (5'-3')
Oligo 1 (SEBF fragments 1 to 4) sense	TTTTGTTCGGATCCTAACCCTTTC
Oligo 2 (SEBF fragments 5 to 7) antisense	CTCCTGGACCCTCGAGCCTTTTCTC
Oligo 3 (SEBF fragments 1 and 10) antisense	GACGAGCTCGAGCTCAATTACACGGATG
Oligo 4 (SEBF fragments 2 and 9) antisense	ACGTAGACTCGAGTGGATCAGTCCATACTC
Oligo 5 (SEBF fragments 3 and 8) antisense	GGCTCGAGCTGGCCCTCAATTCACCCTCAG
Oligo 6 (SEBF fragment 4) antisense	CAAAGACTCGAGGGTCTCAATTGGCATTAC
Oligo 7 (SEBF fragments 5, 8, 9 and 10) sense	ATGGGATCCTGACCTTAAAATCTTTGTTG
Oligo 8 (SEBF fragment 6) sense	GGGATCCCTGGGCCAGCACCACCCAAAAG
Oligo 9 (SEBF fragment 7) sense	GGGAGGATCCACAGTTCCAACAGAGTCTAC
Oligo 14 (Pti4 fragments 1 to 3) sense	GATATACTCGAGGATCAACAGTTACCAC
Oligo 15 (Pti4 fragments 4 to 7) antisense	GTGCTCGAGTCAGACCAATAGTTGATG
Oligo 16 (Pti4 fragments 1, 8 to 10) antisense	CATTCAACTCGAGCCGTCACGGGAAATTC
Oligo 17 (Pti4 fragment 2) antisense	GTCTAACTCGAGTTCAATGTCTTCCCTTC
Oligo 18 (Pti4 fragment 3) antisense	CACCGGCTCGAGTACTCATTCAGGCGCTG
Oligo 19 (Pti4 fragments 4 and 8) sense	ATCCCCTGTGAATTCGAAACATGGGGAG
Oligo 20 (Pti4 5 fragments and 9) sense	GCGCCTGAATTCGTACCTTCTCCGGTG
Oligo 21 (Pti4 fragments 6, 10 and 11) sense	GAAGGGAGAATTCTATAGAGGCGTTAGAC
Oligo 22 (Pti4 fragment 7) sense	CACATTTGGAATTCCCGCACCGGATC
Oligo 23 (Pti4 fragment 11) antisense	GTTTCCTCGAGCCGTTTCACGACGAGCTTAC

Supplemental Table 1. Sequence of the Primers Used for Constructing the Protein Fusions Analyzed in Yeast Two-Hybrid Assays.

CHAPTER 5 – The BTB/POZ of the Arabidopsis Disease Resistance Protein NPR1 Interacts with the Repression Domain of TGA2 to Negate its Function

Contributions

This manuscript, which has been submitted for publication in The Plant Cell, was based largely upon the findings of my primary research project. My direct contributions to this document include Figure 2: A-D, Figure 3: B-D, Figure 4: E Figure 5: C-E, Figure 6: B-F, and Supplemental Figures: 1-2. I was involved in essentially every facet of this work including the development of hypotheses, experiment design, data analysis and the formalization my findings into the following manuscript. I was specifically responsible for writing up the results sections pertaining to the data I contributed and I was also involved in writing the discussion for this manuscript.

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5.1 ABSTRACT

TGA2 and NPR1 are activators of systemic acquired resistance (SAR) and of the SAR marker gene *PR-1* in *Arabidopsis*. TGA2 is a transcriptional repressor, but during SAR, TGA2 recruits NPR1 as part of an enhanceosome. Transactivation by the enhanceosome requires the NPR1 BTB/POZ domain. However, the NPR1 BTB/POZ does not contain an autonomous transactivation domain, and thus its molecular role within the enhanceosome remains elusive. We now show by gel filtration analyses that TGA2 binds DNA as a dimer, tetramer, or oligomer. Using in vivo plant transcription assays we localized the repression domain of TGA2 to the N-terminus and demonstrate by electrophoretic mobility shift assays and a novel technique that it is responsible for controlling the DNA-binding activity of the oligomer both in vitro and in vivo. We confirm that the NPR1 BTB/POZ interacts with and negates the molecular function of the TGA2 repression domain by excluding TGA2 oligomers from cognate DNA. These data distinguish the NPR1 BTB/POZ from other known BTB/POZ and establish its molecular role in the context of the *Arabidopsis PR-1* gene enhanceosome.

5.2 INTRODUCTION

Plant defense against pathogen attack involves global transcriptional reprogramming (Dangl and Jones 2001). Among the induced genes figure the pathogenesis-related (*PR*) genes which are activated both at the site of infection and in uninfected parts of the plant in response to the pathogen-induced accumulation of salicylic acid (SA) (Ryals et al., 1996). Local and distal SA accumulations are mandatory to the deployment of a systemic long-lasting and broad-spectrum plant disease resistance response called systemic acquired resistance (SAR) (Ryals et al., 1996). Exogenous application of SA, termed chemical SAR, triggers *PR* gene induction and SAR deployment (Ward et al., 1991).

Signal transduction downstream of SA requires both NPR1 and the TGA2-clade of transcription factors (Rochon et al., 2006; Zhang et al., 2003; Delaney et al., 1995; Cao et al., 1994). After a rise in SA concentration, nuclear localized NPR1 interacts with TGA2 to stimulate its DNA binding activity to SA-responsive promoter elements, ultimately resulting in the activation of *PR* genes and SAR (Rochon et al., 2006; Johnson et al., 2003; Mou et al., 2003; Zhang et al., 2003; Després et al., 2003; Fan and Dong 2002; Subramaniam et al., 2001; Kinkema et al., 2000; Després et al., 2000; Lebel et al., 1998). Interestingly, TGA2 is a constitutive repressor, but forms an enhanceosome with NPR1, which provides the transactivation domain (Rochon et al., 2006). This domain is located in the C-terminal end of NPR1 and contains cysteines critical to its function (Rochon et al., 2006).

NPR1 contains two identifiable protein-protein interaction motifs: ankyrin repeats (Li et al., 2006; Ryals et al., 1997; Cao et al., 1997) and a BTB/POZ (Broad-Complex,

Tramtrack, and Bric-a-brac/Pox virus and Zinc finger) domain (Aravind and Koonin 1999; Bardwell and Treisman 1994). The ankyrin repeats mediate interactions with TGA factors and their mutation abolishes NPR1-TGA complex formation, *PR* gene expression, and SAR (Després et al., 2003; Després et al., 2000; Zhang et al., 1999; Ryals et al., 1997; Cao et al., 1997). The functional requirements of the NPR1 BTB/POZ in disease resistance are not yet understood. However, this domain is critical to the transactivation function of the TGA2-NPR1 enhanceosome in spite of the fact that it is not an autonomous transactivation domain (Rochon et al., 2006). While in mammals BTB/POZ are actively studied since deregulation of proteins bearing this motif often result in disease states such as cancer (Kelly and Daniel 2006; Collins et al., 2001; Deltour et al., 1999), not much is known about the function of BTB/POZ in plants.

BTB/POZ is an evolutionarily conserved and widely distributed structural motif found in a battery of proteins involved in different biological processes, such as transcriptional regulation, cytoskeletal organization, and formation of voltage-gated channels (Collins et al., 2001; Aravind and Koonin 1999). This domain has been shown to homodimerize, multimerize and heterodimerize with other BTB/POZs or with proteins devoid of the motif (Collins et al., 2001; Aravind and Koonin 1999; Li et al., 1999; Bardwell and Treisman 1994). Although all BTB/POZs identified thus far contain a core of approximately 90 amino acids, a long form of the BTB/POZ also exists which contains an N-terminal extension, approximately 30 residues in length (Stogios et al., 2005). The structure of residues 7 to 122 of the promyelocytic leukemia zinc finger (PLZF) has been solved and offers a three-dimensional view of the long form of the BTB/POZ (Li et al., 1999), the form to which NPR1 likely belongs.

Here, we identify the repression and oligomerization domains of TGA2 and show that the repression domain dictates the stoichiometry of the TGA2-DNA complex. We establish the stoichiometry of the TGA2-NPR1-DNA complex and solve the molecular function of the NPR1 BTB/POZ in the enhanceosome.

5.3 RESULTS

5.3.1 The N-Terminus of TGA2 Is a Non-Autonomous Repression Domain

TGA2 is a constitutive repressor (Rochon et al., 2006) and to identify domains responsible for repression, TGA2 deletions were generated. We focused on the N-terminus (a region arbitrarily defined as the sequence located between the first residue and the basic DNA binding domain) since this is where the transactivation domain is located in tobacco TGA1a (Neuhaus et al., 1994). TGA2 and a variant lacking the first 43 amino acids (Δ43) were respectively fused to the DNA binding domain of Gal4 (:DB) and assayed using an in vivo plant transcription assay system, in which the luciferase reporter gene is first activated by a chimeric LexA:VP16 transcriptional activator (Figure 1B). The baseline level of transcription was determined by transfecting leaves with Gal4 DB (not fused to any other protein or protein domain) along with the reporter construct shown in Figure 1A. To activate the reporter gene, leaves were simultaneously transfected with Gal4 DB and LexA:VP16. In Figure 1B and as reported previously (Rochon et al., 2006), transfection with TGA2:DB and LexA:VP16 resulted in some repression of the reporter gene in resting (white bars) and SA-treated (grey bars) cells. However, expression of the Δ43 variant of TGA2 (Δ43:DB) along with LexA:VP16 did

not result in repression, as values were not below those observed with Gal4 DB + LexA:VP16 and this result was obtained regardless of whether cells were treated with SA. These data indicate that the N-terminus of TGA2 is required for the transcriptional repression properties of TGA2.

We next addressed whether the N-terminus of TGA2 is an autonomous repression domain. The first 47 amino acids of TGA2 were fused to the Gal4 DB (Nt47:DB) and coexpressed with LexA:VP16 (Figure 1B). Whether cells were treated or not with SA, this did not result in repression of the activated reporter gene, as values were not below those observed with Gal4 DB + LexA:VP16. These results indicate that the N-terminus of TGA2 is not an autonomous repression domain.

Since the Δ43 variant of TGA2 and the N-terminus of TGA2 did not repress transcription of the activated reporter gene, we wanted to determine whether they can activate transcription of a reporter construct consisting of a firefly luciferase gene under the control of 5 copies of the Gal4 upstream activating sequences (UAS) fused to a minimal promoter (Rochon et al., 2006). Figure 1C shows results from such an experiment. The baseline level of transcription was determined by transfecting leaves with Gal4 DB (not fused to any other protein or protein domain) along with the reporter construct. Transfection with TGA2:DB or Nt47:DB did not result in reporter gene activation beyond the baseline level, regardless of whether cells were treated with SA (grey bars) or not (white bars). However, transfection of the Δ43 variant of TGA2 (Δ43:DB, Figure 1C) led to SA-independent expression of the reporter gene well above the baseline. These data indicate that removal of the N-terminal repression domain of TGA2 leads to the creation of a protein with transcriptional activating properties.

5.3.2 TGA2 Exists as a Higher Order Complex which Requires the Leucine Zipper

Since the N-terminus of TGA2 is not an autonomous repression domain, we hypothesized that it might play a dynamic structural role in TGA2, imposing a stoichiometry observable in gel filtration or discernible in the context of DNA interaction. We first set out to express soluble TGA2 fused to a C-terminal His-tag in *E. coli* and purify it by affinity chromatography. Purified TGA2 was then analyzed by Sephacryl S300 gel filtration.

The elution profile for TGA2 (Figure 2A) indicates that the protein eluted in the void volume and thus formed an oligomer of unknown stoichiometry, but containing 40 or more units of TGA2, based on the S300 theoretical size exclusion of 1.5 MD and the molecular weight of TGA2 (37.51 kDa with His-tag) (Supplemental Figures 1 and 2). This void volume entity was not constituted of aggregated, non-functional proteins, since it was our source of TGA2 to perform EMSAs and was competent to interact with DNA (Figure 3). An immunoblot analysis with an anti-His-tag antibody confirmed that TGA2 could only be detected in the void volume of the column and could not be found in the included volume (1-column volume) (Figure 2A, inset). Deleting the N-terminal repression domain of TGA2 (Δ43) or the basic DNA-binding domain (Δ68) had no effect on its capacity to form a high order complex as the Δ43 and Δ68 variants also eluted in the void volume (Figure 2B and C). This result was also confirmed by immunoblot (Figure 2B and C, insets). The Δ43 is also constituted of functional proteins and was our source for EMSAs shown in Figure 4. However, further deleting to Δ93, effectively removing the leucine zipper, abolished the formation of the oligomer and resulted in a

species forming mainly a dimer and possibly also a monomer, which can be observed as a slight shoulder around elution volume 70 ml (Figure 2D). The Δ93 elution profile was also confirmed by an immunoblot (Figure 2D, inset). The data indicate that the leucine zipper is required for the formation of a TGA2 oligomer.

5.3.3 The Oligomeric Species of the TGA2 Repressor can Bind to its Cognate Sequence in PR-1

To establish the possible biological significance of the higher order complex form of TGA2, we tested whether the oligomer could bind to cognate DNA elements. To do so, EMSAs experiments were performed using the SA-response element *LS7* (Lebel et al., 1998), which contains a single TGA-binding sequence. The EMSA depicted in Figure 3A indicates that, at low ratios of protein-to-probe, the TGA2-DNA complex formed a single retarded band (solid black arrow), while at higher protein-to-probe ratios, two slower mobility bands (solid grey and open arrow) could also be observed. The very slow migrating band (open arrow) likely represents a high order complex binding to DNA.

As an alternate measure to confirm that TGA2 oligomers could bind DNA, we performed gel filtration experiments in which TGA2 was incubated with a fluorescein-derived *LS7* probe and the elution profile was monitored by fluorimetry. In Figure 3B, the dashed line represents the elution profile of the probe alone (Free DNA), while the solid black line depicts the profile of the double-stranded DNA probe incubated with a high concentration of TGA2 (30 nmol). One peak corresponds to the void volume and contains protein-DNA complexes of undetermined stoichiometry, while the other, based on the standard curve, corresponds to a complex of four TGA2 and one double-stranded DNA

molecules. These results confirm that the TGA2 high order complex binds to DNA and that a TGA2 tetramer can also bind to DNA. An immunoblot confirmed that the elution fractions displaying fluorescence also contained TGA2 (Figure 3C). TGA2 was also observed in fractions corresponding to the dimer; however, this may be the trailing of the tetramer peak. To determine whether TGA2 could bind DNA as a dimer, the probe was incubated with a low concentration of TGA2 (10 nmol). This gave rise to the elution profile represented by a jagged line, which indicates that a TGA2 dimer can interact with one double-stranded DNA. Figure 3D confirmed the presence of TGA2 in the fluorescence-containing fraction. Overall the data presented in Figure 3 indicate that dimers, tetramers, and oligomers of TGA2 can bind cognate DNA.

5.3.4 The N-Terminal Repression Domain of TGA2 Is Mandatory for Binding of the TGA2 Oligomer to DNA in vitro and in vivo

Given that the N-terminus of TGA2 did not manifest itself in a discernible way on gel filtration (profiles from TGA2 or Δ43 are identical), we performed EMSAs using the SA-response element *LS7* (Lebel et al., 1998) to determine whether the repression domain would affect the DNA-binding behavior of TGA2. The EMSA performed with the Δ43 variant of TGA2 (Δ43TGA2) shows that only a single retarded band was observed regardless of the protein-to-probe ratio (Figure 4A). This band co-migrates with the fastest migrating band in the TGA2 EMSA (Data not shown).

To study binding in a more relevant context, we also used the *LS5* to *LS7* region of the *PR-1* promoter as probe (*PR-1*) (Després et al., 2000), which contains two TGA binding elements and compared the binding of Δ43TGA2 (Figure 4B) to that of TGA2

(Figure 4C). As with the *LS7* probe (Figure 4A), binding of the Δ43 variant of TGA2 to the *PR-1* probe showed only a single retarded band, despite the presence of two potential binding sites. In contrast, when the EMSA was performed with TGA2 (Figure 4C), three bands could be observed and presented a pattern of migration similar to what was obtained with the *LS7* probe (Figure 3A). We had previously demonstrated that TGA2 can bind to *LS5* and *LS7*, separately, in EMSAs (Després et al., 2000). In Figure 4D, we performed fluorescence polarization experiments to compare the relative binding affinity of TGA2 towards *LS5* (solid line) and *LS7* (jagged line). The two lines being essentially identical indicate that in vitro, TGA2 cannot discriminate between these two elements of the *PR-1* promoter. The data of Figure 4A-D thus indicate that the N-terminal repression domain of TGA2 controls whether the high order complex can bind to DNA in vitro.

Although there are currently no methodologies to test whether an oligomer forms on DNA in vivo, we set out to address this question by combining chromatin cross-linking, gel filtration, and qPCR. The rational was that if an oligomer forms on *PR1*, we should be able to visualize it by qPCR in the void fraction of an S300 after the chromatin had been cross-linked and sheared by sonication. Figure 4E presents such an experiment and indicate that indeed in wild-type plants (WT), such an oligomer forms on the *PR1* promoter in the absence (open bar), but not after a treatment with SA (grey bar). Next, to demonstrate that this oligomer depends on the presence of TGA2, we repeated the experiment in the tga2/5/6 mutant background (Zhang et al., 2003). Indeed the data indicate that binding of the oligomer to *PR1* is TGA2-dependent. Finally to test whether, in vivo, the N-terminal repression domain of TGA2 was required for the oligomer to bind DNA, as is the case in vitro, the experiment was performed in the tga2/5/6 mutant

background transfected with the Δ43 variant of TGA2. The plant selected showed that the Δ43 variant of TGA2 complements the tga2/5/6 mutation with respect to SA-dependent *PR1* induction (Supplemental Figure 3). The data demonstrates that the oligomer did not form on the DNA when the N-terminus of TGA2 is lacking.

5.3.5 The BTB/POZ Domain of NPR1 Negates the Effects of the N-Terminal Repression Domain of TGA2

TGA2 interacts with NPR1 to form an enhanceosome with transcriptional activation properties requiring the BTB/POZ of NPR1 (Rochon et al., 2006). However, the molecular role of the NPR1 BTB/POZ within the context of the enhanceosome remains elusive. Given that TGA2 is a constitutive repressor (Rochon et al., 2006 and Figure 1B), a hypothetical scenario is that the NPR1 BTB/POZ serves to mask or interfere with the function of the TGA2 N-terminal repression domain. To test this hypothesis, we performed in vivo plant transcription assays and assessed the transactivation capacity of the TGA2-NPR1 enhanceosome using the Δ43 variant of TGA2 (Δ43TGA2:DB) that lacks the N-terminal repression domain, in combination with NPR1 variants mutated in the BTB/POZ (Figure 5A). The rationale for using these mutants has been described in detail (Rochon et al., 2006). The nim1-2 is an NPR1 mutant protein that does not interact with TGA2 (Després et al., 2000) and is shown as a negative control. Deleting the first 22, 44, or 66 amino acids, or removing the core of the BTB/POZ by deleting to amino acid 110 or by substituting it with alanines (A-sub) did not substantially affect the capacity of NPR1 to form an enhanceosome when complexed with Δ43TGA2:DB (after SA treatment). This is in stark contrast with the previous observation that the Δ110NPR1

and the A-subNPR1 did not form a transactivating complex with TGA2, despite their interaction (Rochon et al., 2006).

These results were also confirmed in transgenic plants, where levels of *PR1* transcripts, after SA-treatment, were analyzed by qPCR (Figure 5B). Results indicate that wild-type (WT) plants express higher levels of *PR1* than plants mutated at the npr1 locus (npr1-3) and than plants of the same mutant background (npr1-3) expressing a variant of NPR1 lacking the first 110 amino acids (Δ110NPR1#44) or mutated in the core of the BTB/POZ domain (A-Sub#25). These plants, carrying a non-functional BTB/POZ domain, had previously been described and analyzed (Rochon et al., 2006). Δ110NPR1#44 and A-Sub#25 were used as parent plants for the introduction of the Δ43 variant of TGA2 (Δ43TGA2) and two independent transgenic lines from each genotype (Δ110NPR1#14 and 16 and A-Sub#17 and 19) were selected for qPCR analyses. Results indicate that activation of *PR1* is restored in these lines expressing NPR1 variants carrying a defective BTB/POZ domain when the Δ43TGA2 is co-expressed. The findings of Figure 5A and 5B indicate that the core of the BTB/POZ domain of NPR1 is required for the TGA2 coactivator function of NPR1 only when the repression domain of TGA2 is present in the complex. The corollary is that the BTB/POZ domain of NPR1 negates the function of the N-terminal repression domain of TGA2.

5.3.6 The BTB/POZ Domain of NPR1 Interacts with the N-Terminal Repression Domain of TGA2 to Preclude the Oligomeric Form of TGA2 from Binding to DNA

Since the BTB/POZ domain of NPR1 negates the function of the N-terminal repression domain of TGA2, we hypothesized that these two domains could interact with each other.

To test this potential interaction in the context of DNA binding, we performed a variation on the pull-down assay in which TGA2 was first allowed to interact with cognate DNA (unlabeled version of *LS7* used in the EMSAs of Figures 3 and 4) before addition of the BTB/POZ. Figure 5C indicates that the BTB/POZ domain of NPR1 could indeed be recruited by TGA2 bound to DNA (lane 2, black arrow). A pull-down performed with an *E. coli* extract served as a negative control (Figure 5C, lane 3). The same experiment was then performed with Δ43TGA2. This time, the BTB/POZ could not be detected in the pull-down, indicating that the N-terminal repression domain of TGA2 is essential for recruitment (Figure 5C, lane 6).

Given that Δ43TGA2 cannot bind DNA as an oligomer and that the BTB/POZ interacts with the N-terminus of TGA2, we envisioned a scenario in which the BTB/POZ of NPR1 could possibly prevent the interaction of higher-order forms of TGA2 with DNA. An EMSA was thus performed using the *LS7* probe and in which TGA2 was incubated in the presence or absence of the NPR1 BTB/POZ. Figure 5D shows that incubation of TGA2 with the probe yielded the fast- (black arrow), intermediate- (grey arrow) and slow-migrating (open arrow) protein species binding to DNA (lane 2). However, upon incubation with the BTB/POZ, the only form observed is a migrating species with an intermediate mobility between the fast- and intermediate-migrating bands (asterisk). These results indicate clearly that the presence of the BTB/POZ precludes the oligomer from binding to DNA. The intermediate mobility of the BTB/POZ-TGA2-DNA complex can be interpreted as a supershift of the TGA2-DNA fast-migrating complex and as such, the BTB/POZ would also prevent binding of the intermediate-migrating complex to the probe. A similar experiment was performed with Δ43 (Figure 5E) and shows that

the BTB/POZ had no effect on the binding of Δ43 to *LS7* or the mobility of the Δ43-*LS7* complex.

5.3.7 The TGA2-NPR1 Enhanceosome Has a Stoichiometry of 2 TGA2 to 2 NPR1 to 1 DNA

Knowing that BTB/POZ domains can homodimerize (Melnick et al., 2000; Ahmad et al., 1998; Bardwell and Treisman 1994), we set out to test for NPR1 BTB/POZ dimerization (Figure 6A) as a first step in establishing the stoichiometry of the TGA2-NPR1 complex. To this end, we employed a plant two-hybrid assay system (Rochon et al., 2006; Després et al., 2003). Transfection of the reporter gene and the internal standard along with Gal4 DB served to determine the baseline level of the system. Co-expressing the NPR1 BTB/POZ fused to the VP16 transactivation domain (POZ:TA) with Gal4 DB did not lead to expression beyond baseline. Similarly, expressing the domain fused to Gal 4 DB (POZ:DB) along with the VP16 TA did not activate the reporter gene. However, co-transfecting POZ:DB and POZ:TA lead to a statistically significant ($p<0.05$) increase in normalized luciferase activity indicating that the NPR1 BTB/POZ can self-associate. Association was independent of whether cells were treated (grey bars) or not (white bars) with SA.

The stoichiometry of the BTB/POZ self-association was analyzed by size-exclusion chromatography. To do so, soluble BTB/POZ fused to a C-terminal His-tag was expressed in *E. coli* and purified by affinity chromatography followed by Sephacryl S100 gel filtration. Since this domain could not be expressed to levels sufficiently high for monitoring by absorbance, an immunoblot analysis with an anti-His-tag antibody was

used to detect the presence of the BTB/POZ and confirmed that the domain could self-associate to form a dimer (Figure 6B), based on the theoretical elution profile on an S100 (Supplemental Figures 1 and 2). The dimerization of the domain was also confirmed by cross-linking experiments followed by SDS-PAGE (Figure 6C).

The capacity of the NPR1 BTB/POZ to dimerize would suggest that NPR1 itself could be a dimer under certain conditions. We tested this hypothesis using the plant two-hybrid system (Figure 6D), which monitors interaction inside the nucleus. Co-expressing NPR1 fused to the Gal4 DB (NPR1:DB) with NPR1 fused to the VP16 TA (NPR1:TA) did not indicate that NPR1 could self-associate, as values were not significantly different ($p>0.05$) from expressing NPR1:DB alone and this, whether cells were treated (grey bars) or not (white bars) with SA. We then asked whether TGA2 would have an effect on NPR1 self-association by expressing TGA2, not fused to any domain, with the NPR1:DB-NPR1:TA couple. The results (NPR1:DB + NPR1:TA; black bars) indicate that indeed the presence of TGA2 can enable NPR1:DB-NPR1:TA interaction, since the reporter gene was expressed to higher levels than when NPR1:DB was expressed alone with TGA2 (NPR1:DB; black bars) or when NPR1:DB was co-expressed with NPR1:TA without TGA2 (NPR1:DB + NPR1:TA; grey bars).

We then used gel filtration on Sephacryl S300 to evaluate the stoichiometry of the NPR1-TGA2 complex. The concentration of TGA2 (1 nmol) was set to allow only dimeric species to bind to DNA (Figure 6E; 2 TGA2 + 1 DNA). At these levels, the elution profile cannot be monitored by absorbance. Since NPR1 can self-associate in the presence of TGA2 (Figure 6D), the premise was that the complex would contain two NPR1 bound to a TGA2 dimer interacting with DNA and therefore an equimolar

concentration (1 nmol) of NPR1 was required. Unfortunately, we could not produce such an amount of NPR1 in soluble form. To circumvent this problem, we opted to express a variant of NPR1 mutated in the core of the BTB/POZ through alanine-substitution (Rochon et al., 2006). Although not a perfect solution, since this mutation is known to affect BTB/POZ dimerization (Melnick et al., 2000), this variant yielded a sufficiently high concentration of soluble protein to perform the stoichiometry experiment and an excess of NPR1 (2 nmol) over TGA2 was used. The chromatographic profile indicated that indeed the TGA2-NPR1-DNA complex consisted of two TGA2, two NPR1, and one probe. However, we could also detect a complex containing two TGA2, one NPR1, and one probe (Supplemental Figures 1 and 2). The immunoblot (Figure 6E, inset) confirmed the presence of NPR1 in these fractions. However, NPR1 could still be found in the void volume and in fractions located between the void and the TGA2-NPR1-DNA complex, which suggest that NPR1 was only partially redistributed. When chromatographed on its own, NPR1 elutes exclusively in the void volume (Figure 6F).

5.4 DISCUSSION

Our study has demonstrated that the N-terminal region of TGA2 (amino acid 1-43) contains a repression domain (Figure 1). However, this domain does not act autonomously (Figure 1). Instead, it imparts a certain structure on TGA2, which allows it to bind its cognate DNA sequence as an oligomer of unknown stoichiometry (Figure 3). Removal of this domain precludes TGA2 from interacting with the DNA as an oligomer (Figure 4). However, when TGA2 is removed from the DNA context, the N-terminal

domain of TGA2 does not seem to play a role in the stoichiometry of the factor as, even in its absence (with the deletion of up to the first 68 amino acids), TGA2 remains an oligomer (Figure 2). Instead, the role of oligomer orchestrator seems to fall on the leucine zipper of TGA2 (Figure 2). Since we demonstrated that the NPR1 BTB/POZ interacts with the N-terminus of TGA2, and that a TGA2-NPR1 enhanceosome in which the N-terminus of TGA2 has been deleted no longer requires the BTB/POZ of NPR1 for its transactivation function (Figure 5), our data argue that the BTB/POZ masks or negates the function of the TGA2 repression domain. In addition, since the BTB/POZ precludes oligomeric TGA2 from binding to its cognate DNA sequence (Figure 5), our data further argue that the repressive form of TGA2 might be a higher-order form, while the activating species, that is to say, the one present in the TGA2-NPR1 enhanceosome, may be a dimer or low-stoichiometric form. Thus, after SA-stimulation, which allows TGA2 to recruit NPR1, the BTB/POZ of NPR1 would either disassemble TGA2 oligomers or interact with TGA2 dimers and evict oligomers from the DNA. Regardless of the mechanism, an enhanceosome would result. The presence of a TGA2-dependent oligomer and its disassembly or eviction from *PR1* after SA-treatment is supported by in vivo data (Figure 4E).

Having demonstrated that NPR1 can self-associate in the presence of TGA2 and that the BTB/POZ can dimerize (Figure 6), one can expect the enhanceosome to have a stoichiometry of 2 TGA2:2 NPR1. This stoichiometry is supported by gel filtration experiments (Figure 6). Although, our data also indicate that a stoichiometry of 2 TGA2:1 NPR1 is also possible, this result is plausible but less convincing since it was obtained with a mutated version of NPR1 compromised in the core of the BTB/POZ

domain and therefore, likely to affect dimerization. This mutation, although necessary to obtain sufficient amount of soluble proteins to perform the gel filtration experiment, may have resulted in a less stable TGA2-NPR1 complex.

In EMSA, the leucine zipper of TGA factors is necessary and sufficient for dimerization but a DS (dimer-stabilization) domain, located C-terminal of the leucine zipper, cooperates with it to stabilize the TGA dimer (Katagiri et al., 1992). This has led several groups to conclude without verification that whenever the leucine zipper of a TGA factor is deleted or mutated, dimerization is abolished. However, whether the DS domain can function as an autonomous dimerization interface has never been addressed. Deletion of the first 93 residues in TGA2 (Δ93 constructs in Figure 2) reveals that this construct can dimerize, establishing that the leucine zipper is not required for self-association and suggesting that the DS domain is the primary entity responsible for the dimerization of TGA factors. This result, therefore, opens up the possibility that the leucine zipper may perform tasks other than the well-established dimerization interface. Indeed, progressive deletion of the N-terminus of TGA2 (Figure 2) revealed that TGA2 stopped oligomerizing once the leucine zipper was deleted (deletion of the first 93 amino acids), indicating the importance of this domain in higher-order complex formation. Oligomerization through the leucine zipper is well documented in proteins such as FOXP3 and Translin, where it has been shown that mutations in the domain abolishes oligomer formation (Li et al., 2007; Aoki et al., 1999). In the case of Translin, the protein forms a ring of eight to ten protomers depending on the species of origin (Gupta et al., 2008; Kasai et al., 1997). FOXP3 elutes from gel filtration as a monomer, dimer, tetramer and oligomer. Similarly, and depending on protein concentration, full-length TGA2 can

elute as a dimer, tetramer or oligomer, but smaller species seem to only exist in the presence of cognate DNA (Figure 2A vs 3B).

Concentration-dependent regulation of gene expression by transcription factors has been known for over two decades. A well-documented case is that of the Krüppel zinc-finger protein, which can act as both an activator and repressor on the same DNA element within a promoter depending on protein stoichiometry (Sauer and Jackle 1991). At low concentration, Krüppel binds DNA as a monomer and activates transcription, while at higher concentration, it forms a homodimer and acts as a repressor (Sauer and Jackle 1993). This is reminiscent of TGA2, where the full-length protein, which is capable of binding DNA as an oligomer, represses transcription, while variants lacking this capacity activate transcription. As is the case with Krüppel, TGA2 concentration also dictates which species forms on the DNA. However, even at high concentrations, where oligomers binding to DNA are present, the BTB/POZ domain of NPR1 is capable of either excluding oligomers from forming on cognate DNA or disassembling them altogether (Figure 5C). Thus, the NPR1 BTB/POZ's ability to specifically direct the recruitment of only those TGA2 species competent for gene activation presents a novel means of derepression since this motif typically serves to recruit corepressors (Kelly and Daniel, 2006). Furthermore, the NPR1 BTB/POZ is also atypical in that it not only functions to dismiss or occlude oligomeric TGA2 species from the DNA, but it accomplishes this feat directly rather than through the recruitment of any cofactors. The NPR1 protein appears to marry the duties of derepressor, contributed by the N-terminal BTB/POZ motif, and coactivator, by way of the C-terminal transactivation domain, re-enforcing its role as a key regulator of *PR-1* gene induction.

The data presented here and summarized in Figure 7 constitute a significant advancement to the understanding of the mechanisms by which, domains of the global regulator NPR1 controls *PR-1* gene activation in *Arabidopsis*, and contribute to new insights into the function of BTB/POZ across kingdoms.

5.5 METHODS

5.5.1 Plant Transcription Assays

All procedures for the plant two-hybrid assays were previously described (Després et al., 2003). All constructs were created by PCR as previously described (Després et al., 2003; Rochon et al., 2006). Every bar in each graph represents five bombardments repeated five times (n = 25). *Arabidopsis thaliana* ecotype Columbia was used throughout this study.

5.5.2 Chromatography

His-tagged purified proteins were diluted to the required concentrations in a final volume of 2 ml using S300 running buffer (50 mM HEPES, pH 7.4, 250 mM NaCl) prior to gel filtration analysis on the Sephacryl S100 HR or Sephacryl S300 HR packed in 50 cm long HR 16 columns (GE Health) and equilibrated with S300 running buffer. Elutions, in 0.5 ml fractions, were performed in the same buffer at a flow rate of 0.8 ml/min. Where indicated, proteins were incubated with DNA probes at room temperature in the dark for 20 min prior to chromatography as described above.

5.5.3 EMSAs

Probes were labeled on the 5'-end of each strand with IRDye-700nm (LI-COR). The probes used were the *LS7* probe (5'-TATTTTACTTACGTCATAGATGTGGCGGCA-3' annealed to 5'-TGCCGCCACATCTATGACGTAAGTAAAATA-3') or the *PR-1* probe (5'- GTTTCTCTACGTCACTATTTTACTTACGTCATAGATGTGG-3' annealed to 5'-CCACATCTATGACGTAAGTAAAATAGTGACGTAGAGAAAC-3'). Binding reactions were performed in the dark at room temperature for 20 min in 50 µl of EMSA buffer (20 mM HEPES-KOH, pH 7.9, 250 mM NaCl, 2 mM DTT, 20% glycerol, and 0.5% Tween 20) with 100 fmol of probe prior to loading onto 4% polyacrylamide gels (29.2:0.8 acrylamide-bisacrylamide in 100 mM Tris, 100 mM borate and 10 mM EDTA) and running at 8 V/cm for 70 min. Gels were then scanned on the Odyssey infrared scanner (LI-COR).

5.5.4 Fluorescence Anisotropy

The probes employed were the LS7 probe (5'-TATTTTACTTACGTCATAGATGTGGCGGCA-3' annealed to 5'-FAM-TGCCGCCACATCTATGACGTAAGTAAAATA-3') and LS5 probe (5'-FAM TGACTGTTTCTCTACGTCACTATTTTACTT-3' annealed to 5'-AAGTAAAATAGTGACGTAGAGAAACAGTCA-3'). DNA-binding reactions were performed in the dark at room temperature for 4 h in 100 µl of S300 running buffer containing 0.1 nM probe prior to measurements to allow the reactions to come to equilibrium. The incubation and analysis were conducted in Costar (Corning) 96 well black non-treated round bottom polypropylene plates. Anisotropy determinations were

made with the POLARstar Optima (BMG Labtechnologies) and each reaction was analyzed in quintuplicate.

5.5.5 DNA-Dependent Pull-Down Assays

The biotinylated LS5-LS7 probe (5'-GTTTCTCTACGTCACTATTTTACTTACGTCATAGATGTGG-3'-Biotin annealed to 5'-CCACATCTATGACGTAAGTAAAATAGTGACGTAGAGAAAC-3') was coupled to Streptavidin Iron Oxide Particles according to instructions (SIGMA). His-tagged purified factor (500 pmol), in 400 µl of binding buffer (200 mM NaCl, 1 mM EDTA, 20 mM HEPES pH 7.9), was incubated with the beads for 30 min. at room temperature, with continuous inversion on the roto-torque. The beads were washed twice with 500 µl of binding buffer and once with binding buffer containing 1% milk. Crude *E. coli*-produced cofactor in 500 µl of binding buffer containing 1% milk, was incubated for 30 min. and washed twice without milk as described above. Proteins were eluted by boiling in 45 µl of SDS-PAGE sample buffer and subjected to immunoblot analysis.

5.5.6 qPCR

Total RNA was extracted from leaves using the RNeasy plant mini kit (Qiagen, Mississauga, ON) according to the supplier's instructions. After treatment with DNase I (Invitrogen, Carlsbad, CA), first strand cDNA synthesis was generated using SuperScript II reverse transcriptase (Invitrogen), and the (dT)17VN oligo in the presence of 0.4 U

RNasin (Fisher Scientific, Pittsburg, PA). The newly-synthesized cDNA was diluted 1/200 to reflect a concentration of 10 ng μL-1 input total RNA.

Quantitative Polymerase Chain Reaction (qPCR) was performed on an MX3000 spectrofluorometric thermal cycler (Stratagene, LaJolla, CA) using a two temperature cycling regime initiated with a 15 min activation at 95˚C, followed by 40 cycles of 2 min of annealing and extension at 66 ˚C and 10 sec denaturation at 95 ˚C. Each assay contained 5 ng cDNA, 1 X SYBR Green® (Quantitech; Qiagen), 0.5 pmol oligonucleotides (PR1F 5′-GCTCTTGTAGGTGCTCTTGTTCTTCC-3′ and PR1R 5′-AGTCTGCAGTTGCCTCTTAGTTGTTC-3′), prepared as described in Rutledge and Stewart (2008). The fluorescence data collected at the end of each PCR cycle was analyzed by the absolute quantification via Ct method (Rutledge & Stewart 2008).

5.5.7 Cross-Linking-Chromatography

Plant treatment, cross-linking, sonication, and cross-linking reversal were performed as we do for chromatin-immunoprecipitation (Rochon et al., 2006). Chromatography was as described under "Chromatography". qPCR was performed as we described previously (Gonzalez-Lamothe et al., 2008), with the exception that *PR1* primers (5'-CGCCACATCTATGACG-3' and 5'-GATCGGTCACCTAGAGT-3') and *Ubiquitin5 (UBQ5)* primers (5'-GACGCTTCATCTCGTCC-3' and 5'-GTAAACGTAGGTGAGTCCA-3') were used.

5.5.8 Statistical Methods

All pooled data are expressed as averages and error bars represent ± 1 standard deviation or standard error. When data from two independent populations are compared, statistical significance was assessed using a two-tailed Student *t*-test.

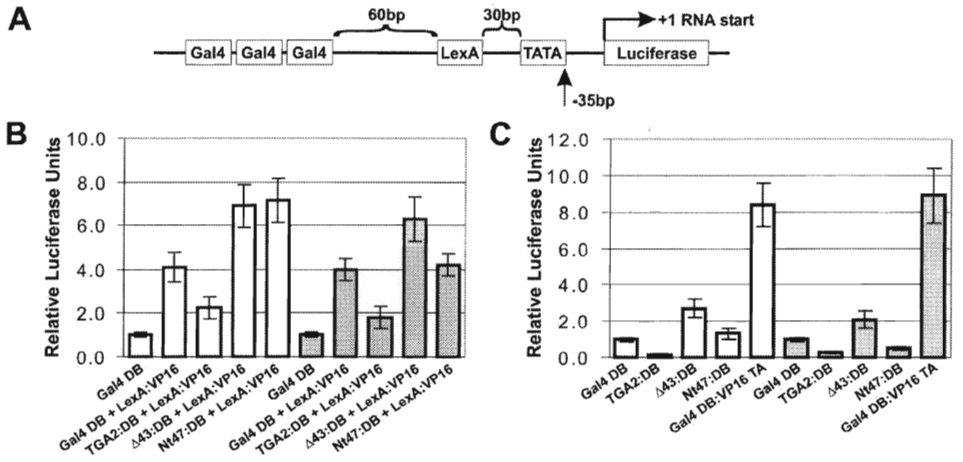


Figure 1. The N-Terminus of TGA2 Is not an Autonomous Repression Domain.

(A) Graphic representation of the synthetic *3X Gal4:1X LexA:minimal promoter:Firefly Luciferase* reporter gene. The upward arrow indicates the position of the TATA box relative to the RNA start site. 60bp and 30 bp indicate the spacing in base pairs between the most downstream Gal4 element and the LexA element and between the LexA element and the TATA box, respectively. Not shown is an omega translational enhancer in the transcribed region of the Luciferase gene.
(B) Bar graph illustrating the assessment of potential transcriptional repression conferred by TGA2, Δ43, and the first 47 amino acids of TGA2 (Nt47), all tethered to DNA through Gal4 DB (:DB). Where indicated, LexA DB fused to the viral particle 16 (LexA:VP16) transactivation domain was also transfected in order to activate the reporter gene. The constructs were transfected along with the *3X* UAS^{GAL4}*:1X LexA DNA element:minimal promoter:*Firefly luciferase reporter and the *CaMV35S*:Renilla luciferase internal standard vectors.
(C) Bar graph illustrating the fact that TGA2 and Nt47 tethered to DNA through Gal4 DB (TGA2:DB and Nt47:DB) do not activate transcription, while Δ43:DB and a chimeric transcription activator composed of the Gal4 DB fused to the transactivation domain of viral particle 16 (Gal4 DB:VP16 TA) do. Gal4 DB represents the baseline level of transcription. The constructs were transfected along with the *5X* UAS^{GAL4}:Firefly luciferase reporter and the *CaMV35S*:Renilla luciferase internal standard vectors. In **(B)** and **(C)** *Arabidopsis* leaves were left untreated (white bars) or were treated for 24 hrs with 1 mM salicylic acid (grey bars). Data are reported as Relative Luciferase Units. Values consist of n=25 samples and represent averages ± 1 SD. Every bar represents five bombardments repeated five times (n = 25).

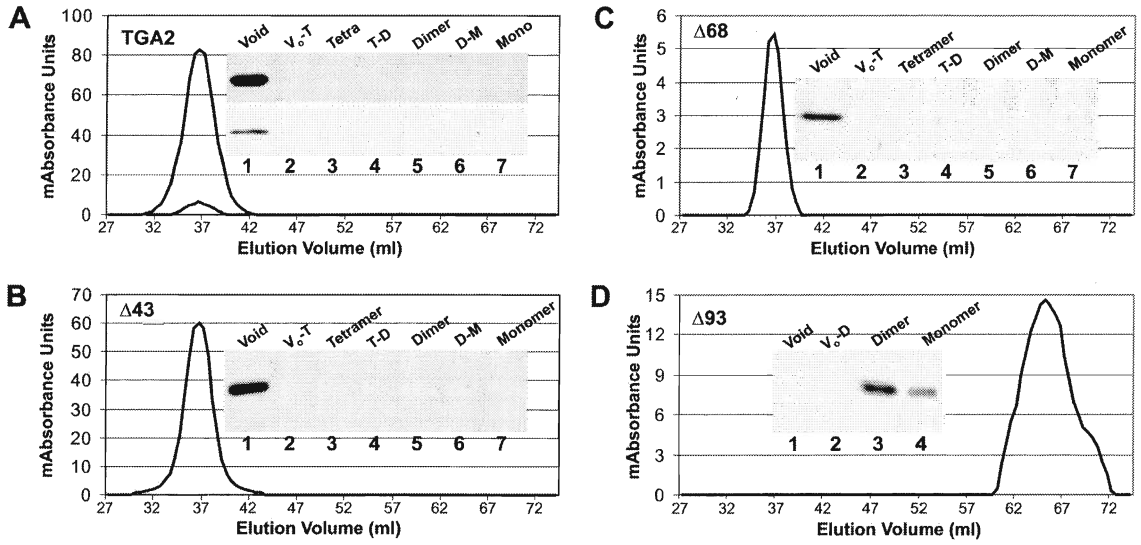


Figure 2. The Leucine Zipper Is Responsible for TGA2 Oligomerization.

Chromatogram illustrating the elution profile of TGA2 **(A)**, Δ43 **(B)**, Δ68 **(C)**, and Δ93 **(D)**. In **(A)**, the higher absorbance curve contains 50 nmol of TGA2 in 2 ml (25 μM), while the lower one contains 5 nmol of TGA2 in 2 ml (2.5 μM). The concentrations of Δ43, Δ68, and Δ93 were 15 μM, 3 μM, and 10 μM, respectively.
In **(A)**, **(B)**, **(C)**, and **(D)** Insets are immunoblot analysis of pooled protein fractions from the chromatogram using an anti-His antibody. In **(A)**, the top and bottom panels represent data from the high and low TGA2 concentration, respectively. Void indicates fractions collected from the void volume, while Tetra, Dimer, and Mono represent pooled fractions from the predicted elution profile of a theoretical TGA2, Δ43, or Δ68 tetramer, dimer, and monomer, respectively. V_0-T, T-D, and D-M indicate pooled samples corresponding to fractions located between the void volume and tetramer, between the tetramer and dimer, and between the dimer and monomer, respectively. In **(D)** V_0-D indicate pooled samples corresponding to fractions located between the void volume and dimer.

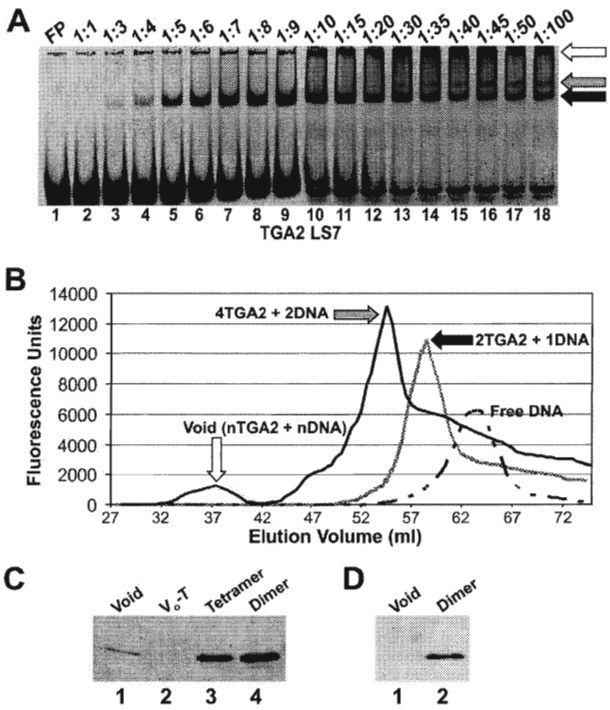


Figure 3. The TGA2 Oligomer can Bind to its Cognate Sequence LS7 in *PR-1*.

(A) EMSA using recombinant TGA2 (lanes 2 to 18) together with the LS7 DNA as the probe (all lanes). The numbers indicate the ratio of probe concentration to TGA2 concentration. The black, grey, and white arrows indicate the position of three distinct complexes. FP stands for free probe and refers to an experiment in which only DNA is present.
(B) Chromatogram based on the elution profile of the LS7 DNA probe derivatized with fluorescein. The profile of free DNA appears as a dashed line, while that of the DNA (5 μM) incubated with 15 μM or 5 μM of TGA2 is represented by a solid or jagged line, respectively. The positions of the maxima correspond to the void volume and to theoretical entities containing four TGA2 and one DNA probes as well as two TGA2 and one DNA probe.
(C) and **(D)** Immunoblot analysis of pooled protein fractions from the chromatogram shown in **(B)**, using an anti-His antibody. The data are from the high **(C)** and low **(D)** TGA2 concentrations. Void indicates fractions collected from the void volume, while Tetramer and Dimer represent pooled fractions from the predicted elution profile of a theoretical TGA2 tetramer bound to two DNA molecules and a TGA2 dimer bound to one DNA molecule. V_0-T indicates pooled samples corresponding to fractions located between the void volume and tetramer.

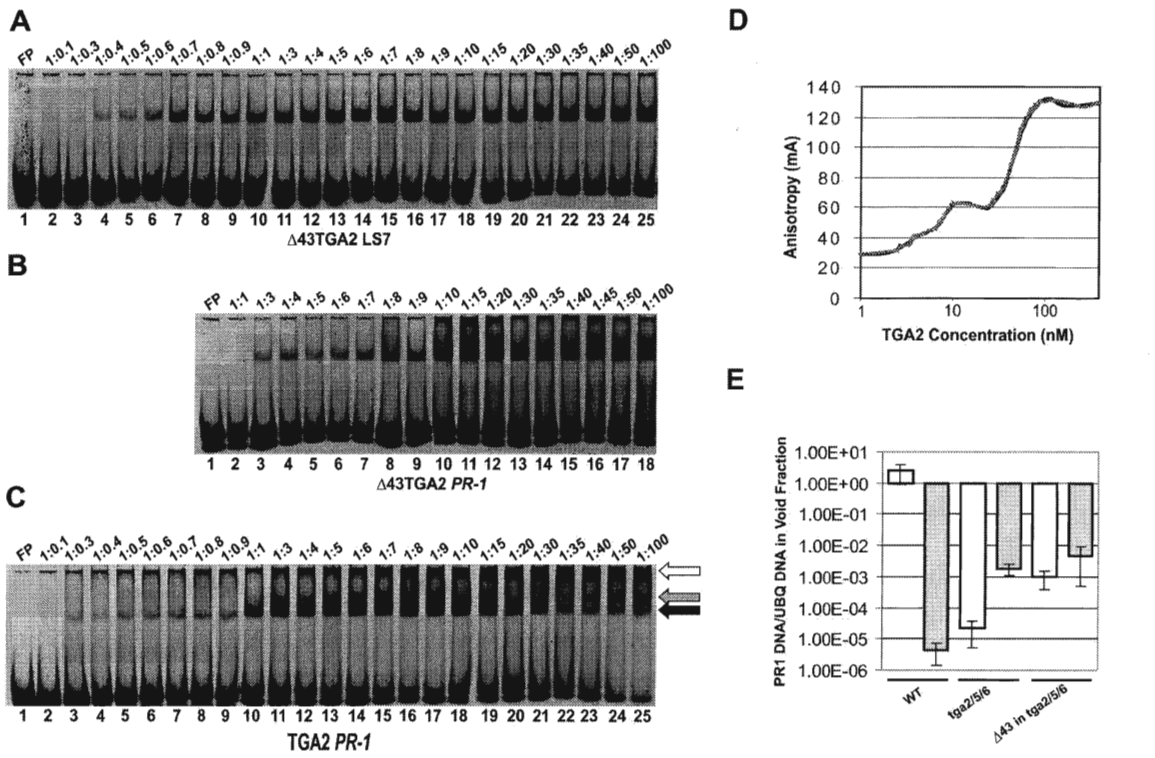


Figure 4. Binding of the TGA2 Oligomer to DNA Requires the N-Terminal Domain of TGA2.

EMSA using recombinant Δ43 **(A)** and **(B)** and TGA2 **(C)** together with the LS7 DNA probe **(A)** or the *PR-1* probe **(B)** and **(C)**. The numbers indicate the ratio of probe concentration to TGA2 concentration. FP stands for free probe and refers to an experiment in which only DNA is present. In **(C)**, the black, grey, and white arrows indicate the position of three distinct complexes.

(D) Fluorescence polarization experiments using recombinant TGA2 together with the LS5 (jagged line) or LS7 (solid line) DNA as the probe. Values are reported as millianisotropy units.

(E) qPCR analyses performed with DNA from wild-type (WT) plants, tga2/5/6 mutants, and tga2/5/6 mutant transfected with the Δ43 variant of TGA2 treated (grey bars) or not (open bars) with SA. Following sonication, the cross-linked chromatin was separated by gel filtration on S300. The void volume was collected, the cross-linking reversed and qPCR was performed using PR1 and ubiquitin (UBQ) primer pairs. Data were reported as the ratio of *PR1* over *UBQ* and represent averages ± 1 SD. Every bar consists of 3 technical replicates on 2 biological replicates (n=6).

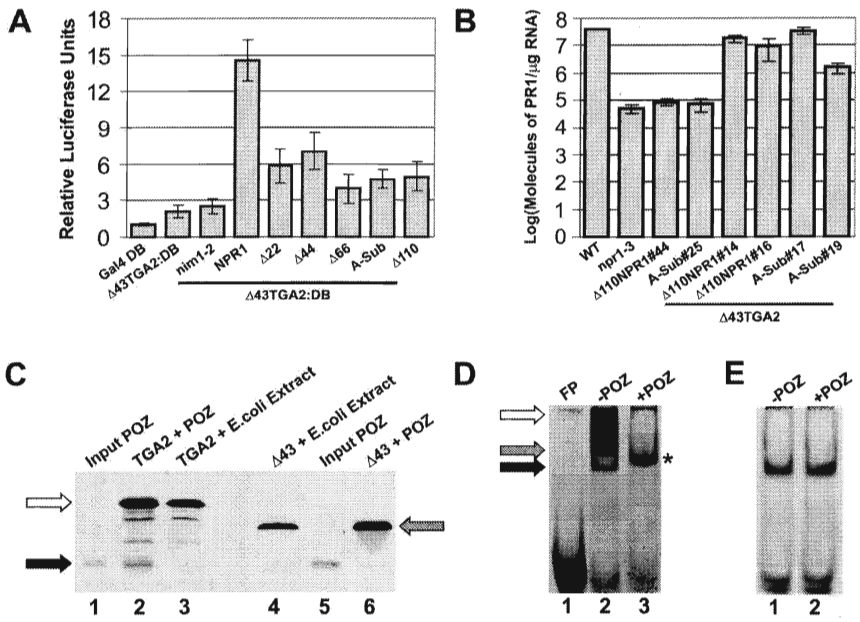


Figure 5. The BTB/POZ of NPR1 Interacts with the N-Terminus of TGA2 and Precludes Binding of the TGA2 Oligomer to DNA.

(A) Bar graph illustrating the transactivation properties of Δ43TGA2 fused to the Gal4 DB in complex with NPR1 and five NPR1 BTB/POZ mutants not fused to any domain. Results obtained with Gal4 DB alone (Gal4 DB) and Δ43TGA2:DB alone are also shown. Δ22, Δ44, Δ66, and Δ110 indicate NPR1 variants in which the first 22, 44, 66, or 110 amino acids have been deleted, while A-Sub refers to an NPR1 variant in which the core of the BTB/POZ has been substituted with alanines (see Rochon et al., 2006 for an in depth rationale of these mutations). Conditions were identical to those described in Figure 1C. Grey bars indicate a treatment with SA. Data are reported as Relative Luciferase Units. Values consist of n=25 samples and represent averages ± 1 SD. Every bar represents five bombardments repeated five times (n = 25).
(B) Bar diagram illustrating the abundance of *PR1* transcript present in wild-type (WT), npr1-3, line 44 of an npr1-3 mutant plant expressing a variant of NPR1 lacking the first 110 amino acids (Δ110NPR1#44), and line 25 of an npr1-3 mutant plant expressing a variant of NPR1 mutated by alanine-substitutions in the BTB/POZ domain (A-Sub#25). Δ110NPR1#44 was used as parent to express a TGA2 variant lacking the first 43 amino acids (Δ43TGA2). Two independent lines from this progeny were tested for *PR1* expression (Δ110NPR1#14and Δ110NPR1#16). Similarly, (A-Sub#25) was used as parent to express a TGA2 variant lacking the first 43 amino acids (Δ43TGA2). Two independent lines from this progeny were tested for *PR1* expression (A-Sub#17 and A-Sun#19). Data for each bar represent averages containing two biological replicates, each composed of six plants. Errors are equal to ± 1 SE. Note that the scale is logarithmic.
(C) Variation of the pull-down assay in which the solid phase was produced by linking biotinylated LS7 DNA to paramagnetic beads followed by binding of TGA2 (lanes 2 and 3) or Δ43TGA2 (lanes 4 and 6) to the DNA. The NPR1 BTB/POZ (lanes 2 and 6) or an *E. coli* extract (lanes 3 and 4) was incubated with the solid phase. Lanes 1 and 5 contain

20% of the amount of BTB/POZ used in lanes 2 and 6. Proteins (TGA2, Δ43TGA2, and POZ) were revealed by immunoblot with an anti-His antibody.
(D) EMSA using recombinant TGA2 (lanes 2 and 3) together with the LS7 DNA as the probe. FP stands for free probe and refers to an experiment in which only DNA was present. POZ indicates that the BTB/POZ of NPR1 had been added (+) or not (-) to the EMSA reaction. The black, grey, and white arrows indicate the position of three distinct complexes. An asterisk denotes the position of a TGA2-BTB/POZ complex.
(E) EMSA identical to that in **(D)** with the exception that Δ43TGA2 replaced TGA2.

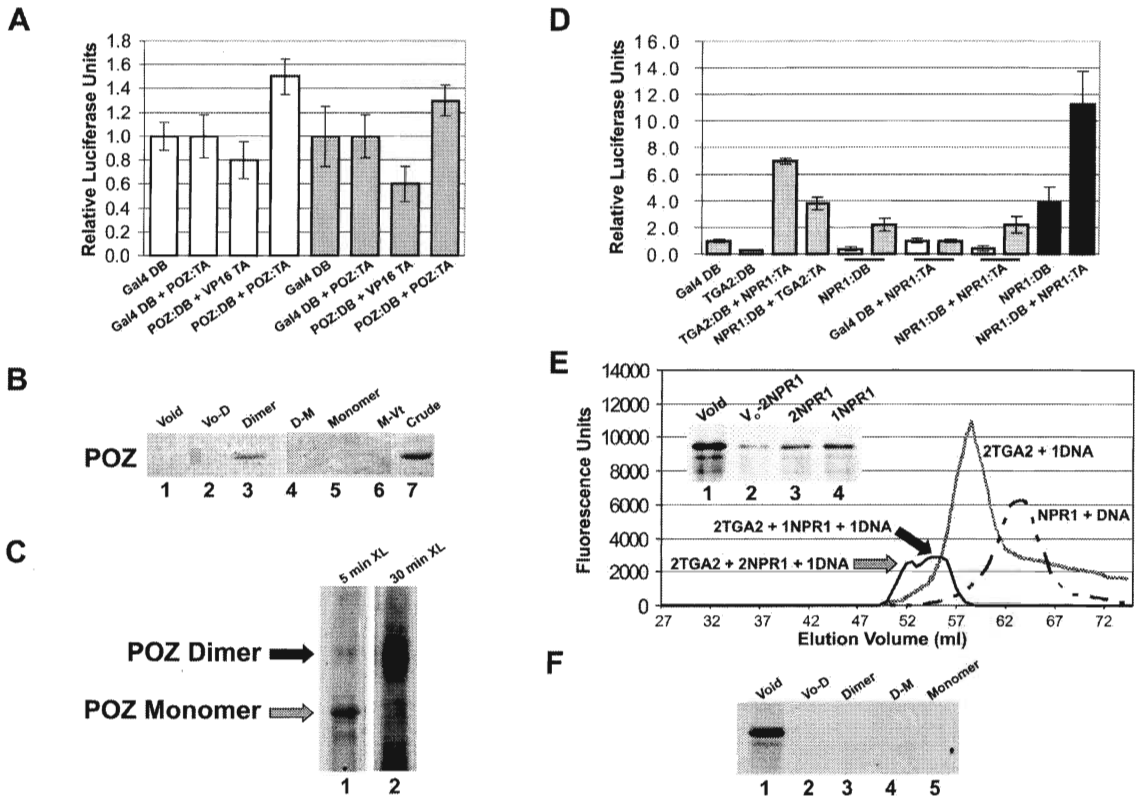


Figure 6. Stoichiometry of the TGA2-NPR1-DNA Enhanceosome.

(A) Bar graph illustrating the self-association of the NPR1 BTB/POZ (POZ) domain. POZ was fused to either the Gal4 DB (POZ:DB) or VP16 TA (POZ:TA). Results obtained with Gal4 DB alone (Gal4 DB) and POZ:DB co-expressed with VP16 TA are also shown for comparison.
(B) Immunoblot analysis of pooled protein fractions from a Sephacryl S100 chromatogram using an anti-His antibody. Void indicates fractions collected from the void volume, while Dimer and Monomer represent pooled fraction from the predicted elution profile of a theoretical NPR1 BTB/POZ dimer and monomer, respectively. V_0-D, D-M, and M-V_t indicate pooled samples corresponding to fractions located between the void volume and dimer, between the dimer and monomer, and between the monomer and one column volume, respectively. Crude refers to a crude *E. coli* extract expressing the NPR1 BTB/POZ domain.

(C) Cross-linking experiment of the NPR1 BTB/POZ followed by SDS-PAGE analysis indicating that the domain dimerizes. Extracts in lanes 1 and 2 were cross-linked for 5 and 30 min, respectively.
(D) Bar graph illustrating that the self-association of NPR1 is dependent on the presence of TGA2. NPR1 was fused to either the Gal4 DB (NPR1:DB) or VP16 TA (NPR1:TA). Results obtained with Gal4 DB alone (Gal4 DB) and TGA2:DB are shown for comparison. TGA2:DB co-expressed with NPR1:TA, and NPR1:DB co-expressed with TGA2:TA are presented to confirm that TGA2 can interact with both types of NPR1 fusion proteins. Black bars indicate experiments following SA treatment in which TGA2 not fused to any domain was also co-expressed. For **(A)** and **(D)**, conditions were identical to those described in Figure 1C. Grey bars indicate a treatment with SA. Data are reported as Relative Luciferase Units. Values consist of n=25 samples and represent averages ± 1 SD. Every bar represents five bombardments repeated five times (n = 25).
(E) Chromatogram based on the elution profile of the LS7 DNA probe derivatized with fluorescein. The profile of free DNA appears as a dashed line (the sample also contained NPR1, which does not interact with the DNA), while that of the DNA incubated with 5 μM of TGA2 (which binds as a dimer under these conditions) is represented by a jagged line. The solid line corresponds to an elution profile in which the sample contained TGA2 (0.5 μM), NPR1 (1 μM), and DNA (0.5 nM). The black arrow corresponds to a theoretical entity containing two TGA2, one NPR1 and one DNA probe, while the grey arrow correspond to one containing two TGA2, two NPR1 and one DNA probe. The inset is an immunoblot analysis using an anti-NPR1 antibody (Després et al., 2000). Void indicates fractions collected from the void volume. 2NPR1 and 1NPR1 correspond to fractions potentially containing an entity composed of two TGA2, two NPR1 and one DNA probe or two TGA2, one NPR1 and one DNA probe, respectively. V_0-2NPR1 indicates pooled samples corresponding to fractions located between the void volume and 2NPR1.
(F) Immunoblot analysis of pooled protein fractions from an S300 elution profile of NPR1 alone using an anti-His antibody. Void indicates fractions collected from the void volume, while Dimer and Monomer represent pooled fractions from the predicted elution profile of a theoretical NPR1 dimer and monomer, respectively. V_0-D and D-M indicate pooled samples corresponding to fractions located between the void volume and dimer, and between the dimer and monomer, respectively.

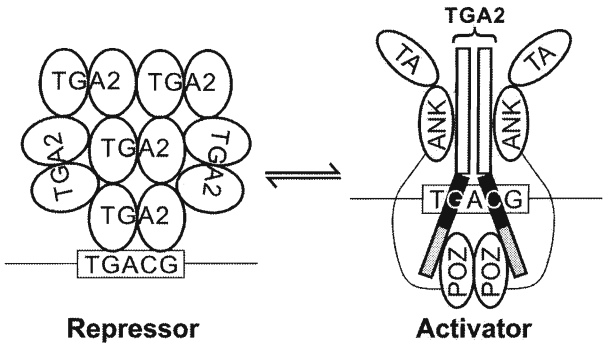


Figure 7. Working Model for the Regulation of TGA2 by NPR1 and Stoichiometry of the TGA2-NPR1 Enhanceosome.

Left panel. In the absence of NPR1 or in resting cells, where NPR1 does not interact with TGA2, TGA2 would form an oligomer capable of binding to its cognate TGACG sequence in the promoter of target genes. This oligomer would repress transcription by a mechanism yet to be identified. Oligomerization of TGA2 on DNA involves the leucine zipper and the N-terminal repression domain.
Right Panel. After a rise in SA and in the presence of NPR1, the NPR1 BTB/POZ (POZ) would either assist in disassembling the TGA2 oligomer or assist in recruiting TGA2 dimers to cognate DNA while excluding TGA2 tetramers and oligomers from binding DNA. The TGA2-NPR1 enhanceosome is likely to have a stoichiometry of 2:2 (TGA2:NPR1). The BTB/POZ domain of NPR1 dimerizes and interacts with the N-terminal repression domain of TGA2 (grey) to mask its capacity to form an oligomer on DNA. The ankyrin repeats (ANK) are the major interfaces stabilizing the TGA2-NPR1 complex, while the C-terminal region of NPR1 contains the transactivation domain (TA) of the enhanceosome.

Supplemental Figure 1. Operational Parameters of the S300 and S100 Gel Filtration Columns.

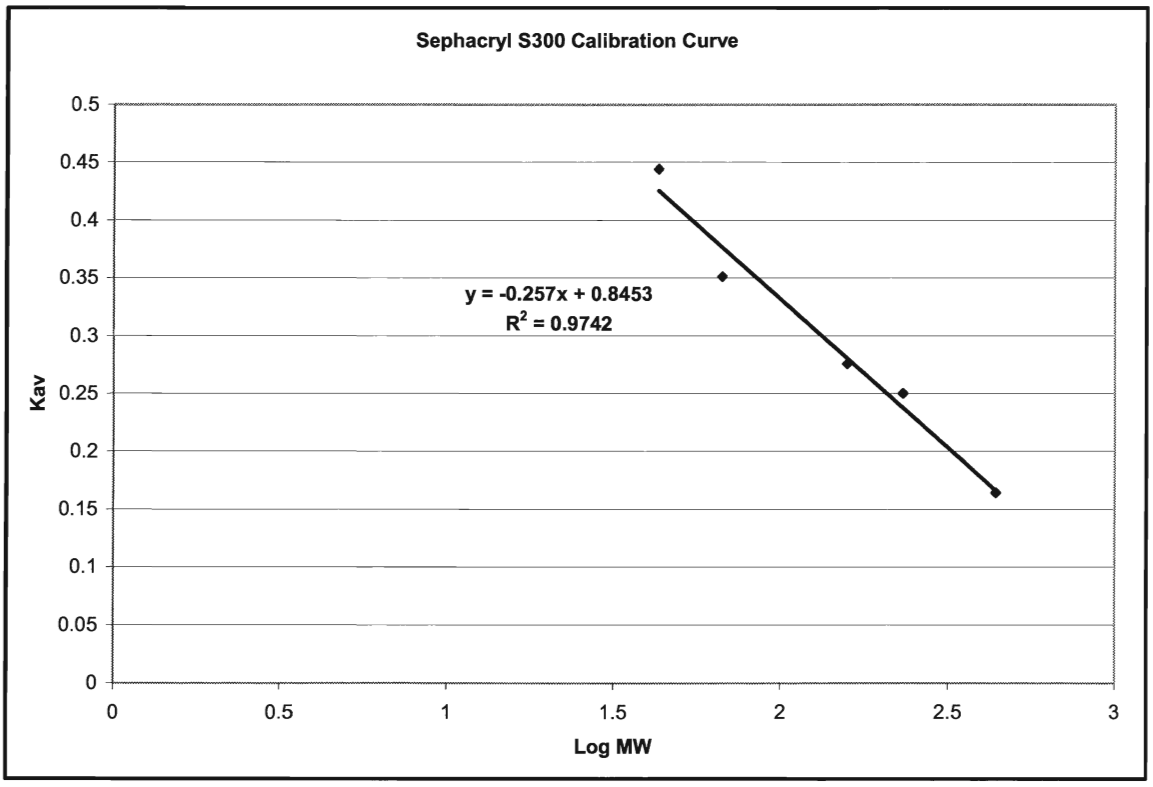
Operational Parameters of the S300 Gel Filtration Column.

Vt (Total bed volume of the column) = 100.5 ml

Vo (Void volume of the column evaluated with Blue Dextran 2000) = 38 ml

Kav = (Ve -Vo)/(Vt-Vo)

MW Standards	kDa	Log MW	Ve (elution volume in ml)	Kav
Ferritin	440	2.643452676	48.27	0.16432
Catalase	232	2.365487985	53.62	0.24992
Aldolase	158	2.198657087	55.234	0.275744
Bovine Serum Albumin	67	1.826074803	59.942	0.351072
Ovalbumin	43	1.633468456	65.774	0.444384



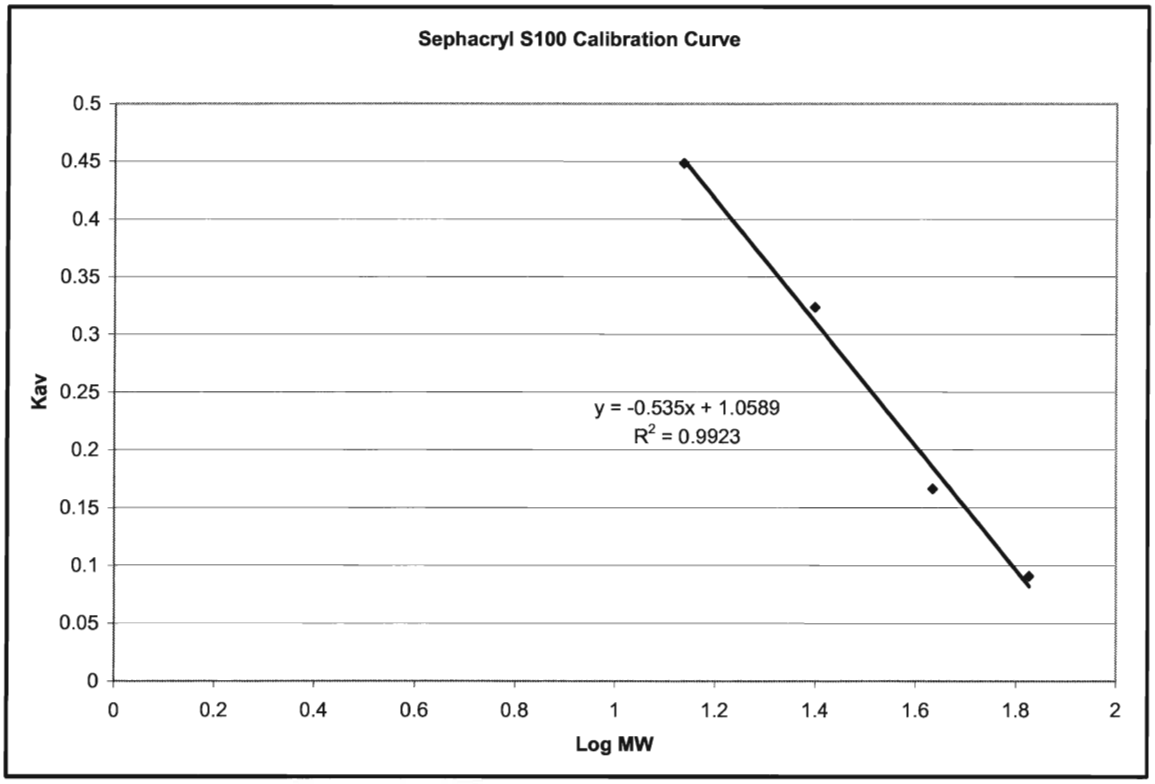
Operational Parameters of the S100 Gel Filtration Column.

Vt (Total bed volume of the column) = 120.64 ml

Vo (Void volume of the column evaluated with Blue Dextran 2000) = 38.393 ml

Kav = (Ve -Vo)/(Vt-Vo)

MW Standards	kDa	Log MW	Ve (elution volume in ml)	Kav
Bovine Serum Albumin	67	1.826074803	45.856	0.090738872
Ovalbumin	43	1.633468456	52.062	0.166194512
Chymotrypsinogen A	25	1.397940009	64.988	0.323355259
Ribonuclease A	13.7	1.136720567	75.264	0.448295986



Supplemental Figure 2. Predicted and Observed Elution Volumes.

Data relating to Figure 2D (S300 Calibration curve).

Anticipated Species	MW (kDa)	LogMW	Kav (Predicted)	Predicted Ve (mL)	Observed Ve (mL)
Δ43TGA2 Δ43TGA2 dimer	27.06 54.12	1.432327792 1.733357788	0.477191757 0.399827049	67.82448484 62.98919053	Peak at 66 ml indicating a mixture of both monomer and dimer

Comments: Molecular weight of proteins includes the His-Tag.

Data relating to Figure 3B (S300 Calibration curve).

Anticipated Species	MW (kDa)	LogMW	Kav (Predicted)	Predicted Ve (mL)	Observed Ve (mL)
Free DNA	18.5	1.267171728	0.519636866	70.47730411	64
1TGA2 + 1DNA	56.01	1.748265573	0.395995748	62.74973424	-
2TGA2 +1 DNA	93.52	1.970904498	0.338777544	59.1735965	59
4TGA2 + 1 DNA	168.54	2.22670299	0.273037332	55.06483323	55
4TGA2 + 2 DNA	187.04	2.271934494	0.261412835	54.33830219	-

Comments:

1) Molecular weight of proteins includes the His-Tag.

2) The anticipated species are based on the fact that fluorescence is the basis of detection and therefore all fluorescent fractions must contain the DNA probe. Secondly, bZIP factors bind DNA as dimers and therefore we should expect TGA2:DNA complexes to bear a protein multiple of 2.

Observations:

1) The free DNA migrates faster than theoretically expected. This is due to the fact that it elutes very late in the chromatography where linearity is more of an issue.

2) There are no elution peaks corresponding to a TGA2 monomer bound to a single DNA probe (1TGA2 + 1DNA) or to a TGA2 tetramer bound to two DNA probes (4TGA2 + 2DNA).

3) The observed elution peaks at 59 and 55 ml would support protein:DNA species containing a TGA2 dimer bound to a single DNA probe (2TGA2 + 1DNA) and a TGA2 tetramer bound to a single DNA probes (4TGA2 + 1DNA).

Data relating to Figure 6B (S100 Calibration curve).

Anticipated Species	MW (kDa)	LogMW	Kav	Predicted Ve (mL)	Observed Ve (mL)
BTB/POZ	21.9	1.340444115	0.341762399	59.36014991	-
BTB/POZ dimer	43.8	1.641474111	0.180711351	49.29445943	49

Comments:

1) Molecular weight of proteins includes the His-Tag.

2) The amount of NPR1 BTB/POZ that could be produced and loaded on the column was below the detection level provided by the UV-absorbance detector. Proteins were monitored by immunoblot using an anti-His-Tag antibody.

Anticipated Species	MW (kDa)	LogMW	Kav	Predicted Ve (mL)	Observed Ve (mL)
1NPR1 + 2TGA2 +1DNA	160.38	2.2051502	0.278576	55.41102477	55
2NPR1 + 2TGA2 +1DNA	227.2	2.3564083	0.239703	52.98144125	52.5

Data relating to Figure 6E (S300 Calibration curve).

Comments:

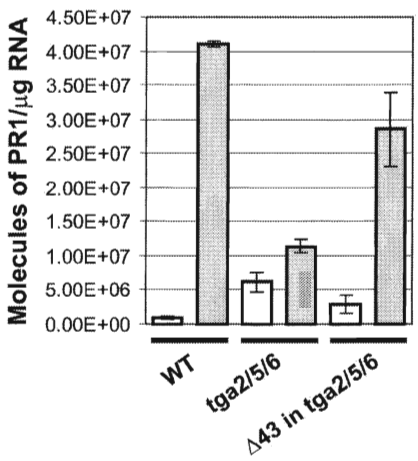
1) Molecular weight of proteins includes the His-Tag.

2) The anticipated species are based on the fact that fluorescence is the basis of detection and therefore all fluorescent fractions must contain the DNA probe. Secondly, bZIP factors bind DNA as dimers and therefore we should expect TGA2:DNA complexes to bear a protein multiple of 2.

Observations:

The observed peaks are separate but overlapping, supporting a model in which one or two NPR1 molecule would interact with the TGA2 dimer bound to a single DNA probe. The presence of NPR1 in these peaks was confirmed by immunoblot using an anti-NPR1 antibody.

Supplemental Figure 3. Quantitation of *PR1* mRNA in the Wild-Type, tga2/5/6 Mutant and tga2/5/6 Mutant Plants Expressing the D43 variant of TGA2.



Supplemental Figure 3. Bar diagram illustrating the abundance of *PR1* transcript present in wild-type (WT), tga2/5/6, and tga2/5/6 mutant plant expressing a TGA2 variant lacking the first 43 amino acids (Δ43TGA2). Grey bars indicate that the plants have been treated with SA, while the absence of treatment is denoted by with bars. Data for each bar represent averages containing two biological replicates, each composed of six plants. Errors are equal to ± 1 SE.

CHAPTER 6 – GENERAL DISCUSSION AND CONCLUSIONS

The plant immune response involves global transcriptional reprogramming. It is understood that the mass activation of defense genes is critical to plant disease resistance, however little is known of the mechanisms that regulate the deployment of defense genes. Our current knowledge of the plant immune system has evolved primarily through the use of genetic approaches. While such strategies have succeeded in identifying the components involved in disease resistance, they offer little insight as to how these components combine to control defense gene induction. The studies presented in this thesis employed predominantly molecular and biochemical methods to further our understanding of how cofactors, transcription factors and their cognate regulatory sequences interact to collectively govern the expression of the *Arabidopsis PR-1* and potato *PR-10a* genes, under both resting and inducing conditions. These efforts also demonstrated that inducible defense genes from the model system, *Arabidopsis*, and the crop species, potato, while activated under similar circumstances, are regulated by vastly different means.

6.1 The *PR-1* and *PR-10a* are maintained in a repressed state under resting conditions

Regulation of the immune responses is a common theme across eukaryotes because the deployment of defensive mechanisms is costly, and often harmful to organism itself. Mutant plants with constitutively activated defense programs commonly demonstrate decreases in their fertility and seed set, in addition to spontaneous cell death (Heidel et

al., 2004). For these reasons, it is not surprising that the promoters of the *PR-1* and *PR-10a* are both occupied by transcriptional repressors under non-inducing conditions, however what is interesting are the considerably different means by which these related genes are negatively regulated.

The TGA2-clade of transcription factors are required for the basal repression of *PR-1* (Chapter 3, henceforth referred to as Rochon et al., 2006). The first indication that the TGA2-clade of transcription factors were involved in the repression of *PR-1* was demonstrated by the elevated level of *PR-1* expression in the tga2/5/6 triple knock out plant under resting conditions (Zhang et al., 2003). However, genetic and molecular approaches suggested that TGA2 was a transcriptional activator that was recruited to the promoter in an SA- and NPR1-dependent manner (Fan and Dong, 2002; Zhang et al., 2003; Johnson et al., 2003). The mechanism preventing the spurious expression of *PR-1* remained elusive because it is difficult to envision a mechanism by which TGA2 could mediate *PR-1* repression if the factor is not present at the promoter under non-inducing conditions. The unquestionable role of the factor in *PR-1* activation also cast doubt on the repression function of TGA2.

We were able to definitively demonstrate that TGA2 was a constitutive transcriptional repressor through the use of an *in planta* transcription assay (Rochon et al., 2006). This system showed that TGA2 could repress an activated reporter gene through the heterologous GAL4 DB in resting or SA-stimulated leaves. This system was further used to demonstrate that the native TGA2 factor could also repress reporter gene expression in the context of the *PR-1* promoter and that this repression function was independent of SA treatment (Rochon et al., 2006). These findings suggest that the

conformation adopted by the factor upon binding its cognate cis-element through its endogenous DB, or that produced upon recruitment to the GAL4 UAS (upstream activating sequence) through the heterologous GAL4 DB, are both sufficient to mediate the active repression of the reporter gene. However, it is not known if this repression is conducted by way of a conserved mechanism.

We also established that *in planta* TGA2 was recruited to the *PR-1* promoter in an SA- and NPR1-independent manner using the ChIP procedure (Rochon et al., 2006). The *in planta* transcription assay and ChIP data, supported by the *PR-1* derepression observed in the tga2/5/6 mutant, demonstrated that the negative regulation of the *PR-1* was indeed controlled by the TGA2-clade of transcription factors. We have gone on to establish that TGA2-mediated repression is contingent upon the factor's ability to form oligomeric complexes on cognate DNA elements (Chapter 5). This oligomeric conformation enables the TGA2-clade of transcription factors to form a high molecular weight complex on the *PR-1* promoter under resting conditions (Chapter 5: Figure 4), but is it unclear how this complex operates to repress transcription. Since the TGA2 N-terminal motif did not behave as an autonomous repression domain (Chapter 5: Figure 1), it seems unlikely that TGA2-mediated repression proceeds through the recruitment of a corepressor. It is however possible that the corepressor recruitment interface is only manifested by the oligomeric conformation of TGA2. It could also be speculated that the TGA2-dependent oligomer simply functions to occlude the transcriptional machinery from the promoter and in doing so prevents aberrant *PR-1* expression. Such a model has been proposed for the oligomeric TEL transcriptional repressor (Kim et al., 2001). The TEL has been shown to effect reporter gene repression when operating from a DNA element up to 600 base

pairs upstream of the transcriptional start site (TSS) (Fenrick et al., 1999). The TGA2 cognate DNA elements in the *PR-1* promoter are situated between 600-700 base pairs upstream of the TSS, further supporting the possibility of a TEL-type oligomer-mediated repression mechanism. Interestingly, replacement of the TEL homo-oligomerization domain with a bZIP motif (TGA2-type DNA binding domain and oligomerization motif) did not affect the capacity of the factor to repress reporter gene activity (Kim et al, 2001).

The two TGA-binding sites in the *PR-1* promoter reside within cis-elements that possess contrasting functions. This arrangement presents an interesting regulatory situation, particularly in light of the high molecular weight complex we now know occupies the promoter under resting conditions. The LS7 promoter element is required for SA-inducible *PR-1* activation, whereas the LS5 negatively regulates *PR-1* expression before and after SA-treatment (Lebel et al., 1998). Unfortunately, due to limits in the resolution of the ChIP assay, we cannot resolve which of the sites are bound by the TGA2/5/6-oligomer (Rochon et al., 2006). Fluorescence anisotropy and EMSA studies indicate that the TGA2 has equal affinity for both the LS7 and LS5 sites in vitro (Chapter 5: Figures 3 and 4), and given that these elements are separated by a mere 20 nucleotides (Lebel et al, 1998), we cannot rule out the possibility that both positions are occupied by the oligomeric complex. In vivo foot printing experiments performed on the *PR-1* promoter found that under non-inducing conditions the LS7 is protected (Lebel et al., 1998). This protection may well be the result of the TGA2/5/6-oligomer. It could be reasoned that the TGA2/5/6-oligomer effects *PR-1* repression by obstructing this positive cis-acting element.

There is some evidence that basal repression of *PR-1* is also mediated in part by histone modifications. The SNI1 protein is proposed to function as a scaffolding protein recruiting various chromatin modifying activities (Mosher et al., 2006). Plants bearing an *sni1* mutation demonstrate increased basal *PR-1* expression (Li et al., 1999; Mosher et.2006). The elevated basal expression of *PR-1* in the *sni1* mutant is attributed to modest increases in H3K4 methylation and general acetylation at H3 at the promoter (Mosher et al., 2006). This explanation conflicts somewhat with the findings of Koorneef et al., (2008), which reported *PR-1* could be activated without significantly altering the general acetylation of H3 at the promoter. These results suggest that H3 acetylation does not control *PR-1* expression. Such data coupled with the very mild changes in histone marks observed in the *sni1* mutant do not make a convincing case that the factor negatively regulates *PR-1* expression through chromatin modifications. However it is not possible to discount that histone modifications contribute to the repression of the *PR-1*. It is widely acknowledged that the chromatin context is critical to basal transcriptional repression in eukaryotes and it undoubtedly plays a role in the negative regulation of *PR-1*, but the current studies have yet to identify the critical histone marks and chromatin modifying agents involved.

In resting cells, the NPR1 protein is localized to both the nucleus and the cytosol (Després et al., 2000; Spoel et al., 2009). Through the use of the ChIP technique, we revealed that the NPR1 coactivator is specifically present in the regulatory region of the *PR-1* gene under non-inducing conditions, and further demonstrated that the recruitment of the coactivator to this locus is independent of the TGA2 clade of transcription factors (Rochon et al., 2006). NPR1, like most coactivators, lacks a known DB domain and is

therefore likely to be maintained at the repressed *PR-1* promoter by way of another protein. However, there is currently no information as to what the NPR1-anchoring entity might be, nor is there any indication of the function of NPR1 in this non-inducing situation. The presence of other coactivators at unactivated or repressed promoters, although uncommon, has also been reported in Drosophila using the ChIP technique (Martinez and Arnosti, 2008). The presence of coactivators such as NPR1 at repressed promoters does not conform to the existing paradigm for gene regulation. However, it could be reasoned that the proximity of these latent coactivators to cis-regulatory elements renders them perfectly poised to activate gene expression in response to the appropriate cue. Despite the presence of NPR1 at the *PR-1* promoter in resting cells, *npr1* mutations do not affect the basal repression of the locus (Cao et al., 1998).

Using the ChIP technique, we established that the *PR-10a*, like the *PR-1*, is bound by a transcriptional repressor, SEBF, under resting conditions (Chapter 4, henceforth referred to as Gonzáles-Lamothe et al., 2008). However, unlike the situation at *PR-1* in which knock out of the TGA2-clade resulted in *PR-1* derepression, knocking down the expression level of the SEBF did not derepress the *PR-10a* (Gonzáles-Lamothe et al., 2008). The lack of derepression observed in the SEBF RNA-interference (RNAi) lines could be due to residual expression of SEBF, but such an explanation seems doubtful because the ChIP assays indicated that SEBF protein was absent from the *PR-10a* promoter in the RNAi lines. It is possible that the SEBF contributes to the negative regulation of the *PR-10a* by altering local DNA or chromatin structures rather than directly preventing the recruitment of the basal transcriptional machinery. In such a case the absence of SEBF would render a more permissive promoter conformation, however

this relaxed structure alone is not sufficient to allow for the expression of the *PR-10a*. The more permissive promoter architecture might account for the enhanced levels of *PR-10a* expression reported following wounding and elicitation in the SEBF knock down tissues (Gonzáles-Lamothe et al., 2008). Despite the fact that under non-inducing conditions the TGA2-clade and SEBF are both clearly negative regulators of transcription at their respective promoters, the means by which these factors contribute to the repression of the *PR-1* and *PR-10a* differ considerably.

The *PR-1* and *PR-10a* promoters appear to be much different doorways for the transcriptional machinery. The former is presumably double stranded DNA in which the major regulatory elements are located between –600 and –700 base pairs from the transcriptional start site (TSS), while the latter appears to be a ssDNA locale with all characterized cis-acting elements no more than 200 nucleotides upstream of the TSS. The SE element, to which the SEBF binds, resides immediately upstream of the TATA box at the *PR-10a* (Boyle and Brisson, 2001). The occupation of the SE by SEBF could potentially function to impose DNA strand separation that extends into the proximal TATA box, producing a conformation which cannot be recognized by the basal transcriptional machinery. Similar repression mechanisms have been proposed for the repression mediated by the Purα and Purβ ssDNA-binding proteins in the mammalian system (Knapp et al., 2006).

It is important to note that at present it is not known if the *PR-10a* promoter is in any way single-stranded. The *PR-10a* promoter is presumed to exist in this uncommon conformation because the two major transcriptional regulators of this gene, SEBF and Why1, are ssDNA-binding proteins in vitro (Després et al., 2000; Boyle and Brisson,

2001). There are reported instances in which ssDNA-binding proteins are capable of binding duplex DNA-elements in concert with a cofactor complex (Mahajan et al., 2005). Since the SEBF is known to complex with the Pti4 in the context of DNA, it is possible that this unlikely cofactor could enable the SEBF-Pti4 repressosome to bind double-stranded DNA (for further discussion on this particular matter see Gonzáles-Lamothe et al., 2008).

Another interesting and entirely uninvestigated aspect of the *PR-10a* promoter is the local chromatin structure. While there is some precedent for chromatin intermitted with patches of ssDNA, this is not a common occurrence and is it predominantly associated with active transcription (Michelotti et al, 1996; Ronai et al., 2007). There is also some evidence that suggests chromatin associated ssDNA functions in DNA recombination events (Ronai et al., 2007). Interestingly, pathogen-induced systemic plant defense signals have been shown to stimulate DNA rearrangements (Kovalchuk et al., 2003). At present it is unknown if any such phenomena occur at the *PR-10a,* but these prospects warrant investigation. It is important that we examine if the promoter region is comprised of ssDNA elements, and if this region is in anyway chromatinized in order to further our understanding of *PR-10a* regulation and investigate the potential role of pathogen-induced DNA recombination at this defense response locus.

To date the only characterized cofactor at the *PR-10a* locus under resting conditions is the Pti4. Unlike NPR1, which does not have an obvious function in the basal repression of *PR-1* despite the fact the coactivator is present at the locus under non-inducing conditions, the Pti4 is essential for the recruitment of the SEBF transcriptional repressor to the *PR-10a*. The Pti4 is known to function as a transcriptional activator.

However because this transcription factor is recruited to the *PR-10a* by way of its association with the SEBF, the Pti4 would be considered a cofactor, more specifically a corepressor. Understanding that the Pti4 serves as a transcriptional activator in various contexts and a corepressor at the *PR-10a* suggests the factor might be involved in a transrepression-type mechanism, a phenomenon observed in the negative regulation of immunological programs in mammalian systems (Rosenfeld et al., 2006). In a typical transrepression scenario, a transcriptional activator is modified, by way of ligand binding or post-translational modification, directing the factor to a different promoter where it binds to previously recruited corepressor complexes preventing their clearance or eviction from the locus (Rosenfeld et al., 2006). As a result the corepressor complexes are secured at the promoter and function to maintain basal repression of the gene (Rosenfeld et al., 2006). It is of course impossible to determine if Pti4 participates in transrepression events without knowing the other promoters to which Pti4 is recruited in potato and the effect of the factor at these loci, but it remains an interesting prospect.

The research conducted during this thesis project has established the means by which the agents and elements present at the promoter combine to negatively regulate expression of both the *Arabidopsis PR-1* and potato *PR-10a* under resting conditions. A clear conclusion from this investigation is that the basal repression of *PR-1* and *PR-10a* are mediated by much different means.

6.2 The activation of the *PR-1* and *PR-10a* proceed through different mechanisms

Transcriptional reprogramming is critical to the deployment of plant inducible defenses. Both *PR-1* and *PR-10a* are activated as components of defensive programs in *Arabidopsis* and potato, respectively. Despite the fact that these PR genes are expressed under similar circumstances, the research conducted in this thesis project has demonstrated that the mode of induction is not conserved between the *PR-1* and *PR-10a*.

In response to SA we have established that the TGA2-clade of transcription factors retain their capacity for repression (Rochon et al., 2006), but interaction with NPR1, specifically involving the BTB/POZ domain, alters the conformation of TGA2 at the *PR-1* promoter (Chapter 5: Figure 4). The repressive oligomeric TGA2 complex is cleared or redistributed such that only a low order TGA2 structure, presumably a dimer, is present at the promoter in the context of an NPR1-TGA2 transactivating complex with a likely stoichiometry of 2 NPR1:2 TGA2 (Rochon et al., 2006; Chapter 5: Figure 6). While it is tempting to believe that in an activating situation the TGA2 is localized to the positive cis-acting *PR-1* element LS7, it is impossible to specify the location of the factor on the promoter due to the resolution limits of the ChIP procedure.

In our efforts to advance the mechanistic understanding of *PR-1* gene activation we have demonstrated that the TGA2-clade of factors are required for the *PR-1* repression under resting conditions and are also essential for *PR-1* activation following SA stimulation. Importantly, the capacity of TGA2 to mediate repression is not directly affected by treatment with SA (Rochon et al., 2006). In the absence of a functional NPR1, TGA2 continued to repress in the context of both the heterologous GAL4 UAS and *PR-1* promoters in SA-stimulated tissues (Rochon et al., 2006). Such observations cast some doubt on the possibility that the capacity of TGA2 to activate or repress

transcription is modulated by a simple switch-type mechanism mediated by a post-translational modification, stimulated by SA treatment. Further supporting this viewpoint is a previous study that demonstrated the TGA2 transcription factor is phosphorylated by a CK2-type kinase activity, which emerges in response to SA stimulation (Kang and Klessig, 2005). However, mutation of the phosphorylated residues in TGA2 did not affect the ability of the factor to activate *PR-1* expression following SA treatment (Kang and Klessig, 2005). The activator function of TGA2 is only realized when complexed with NPR1 (Rochon et al., 2006). However, the TGA2 activator function is not confined solely to the *PR-1* promoter, since TGA2 can also activate gene expression in a heterologous context through the GAL4 DB in an SA-and NPR1-dependent manner (Fan and Dong 2002; Rochon et al., 2006). The ability of TGA2 to manifest repressor/activator duality in these two unrelated contexts might suggest that the function of TGA2 is modulated minimally through DNA-binding allosteric effects. It is quite possible that the DNA binding mediated through its endogenous DB domain, or by way of the GAL4 DB domain, produces equivalent changes in the NPR1-TGA2 complex conformation, resulting in a common means of activation. However, it is not possible to rule out the prospect that the complex adopts different conformations in these two contexts, and that activation proceeds through different mechanisms. In this case, despite the dramatic difference in conformation changes imposed by the binding of different cis-elements, TGA2 would still be able to maintain the motifs required to mediate repression and those necessary to recruit NPR1, ultimately effecting activation. The ability of TGA2 to maintain these interaction interfaces in different DNA contexts would enable the factor to retain its transcriptional duality.

The activation of the *PR-10a* is a much different event than that of the *PR-1,* because unlike the *PR-1,* the activating agents are not constitutive residents at the *PR-10a* promoter. Under resting conditions the *PR-10a* promoter is bound by the SEBF-Pti4 repressosome (Gonzáles-Lamothe et al., 2008). Upon wounding or elicitation these negative regulators are cleared or dismissed, and the activator Why1 is recruited to the ERE promoter element at the *PR-10a.* Despite the unusual ssDNA-binding nature of the factors responsible for regulating expression of the *PR-10a,* this gene is controlled in a far more conventional fashion than that of the *PR-1.* In the case of the *PR-10a,* repression and activation are effected through entirely different repressors and activators, which are recruited in a mutually exclusive manner to the promoter where they occupy distinct negative and positive DNA elements, respectively. The situation at the *PR-1* is much different because the TGA2 functions in both repression and activation and the NPR1 coactivator is present at the promoter under non-inducing conditions.

Based on the current data, the *PR-1* and *PR-10a* promoters seem to be much different environments under activating conditions. Unfortunately, very little is known about the actual DNA and chromatin architectures at either locus under these circumstances. Limited investigations into the histone marks associated with *PR-1* expression have been conducted but they are in many ways conflicting, and as a result it remains unclear how such modifications contribute to the activation of this *PR* gene (Mosher et al., 2006; Koorneef et al., 2008; van den Burg and Takken, 2009). This aspect of *PR-10a* regulation remains entirely unaddressed, but it would be very interesting to determine if this seemingly single-stranded locus is capable of supporting nucleosomes under any conditions.

The Why1 activator provides an interesting link between the mechanisms of activation for the *PR-1* and *PR-10*. The *Arabidopsis* Why1 ortholog (AtWhy1) is required for the activation of *PR-1* and the inducible defense response program SAR (Desveaux et al., 2004). EMSA analysis also demonstrated that the binding activity of the AtWhy1 for the 3' portion of the ERE, termed the PBF-2 binding (PB) element, was stimulated in an SA-dependent, NPR1-independent manner. Using the ChIP method we have now shown that the AtWhy1 is recruited to the *PR-1* promoter, presumably to a PB element occurring between – 879 to – 872, in an SA-dependent manner (Boyle and Després, unpublished data). The recruitment patterns of the potato Why1 (StWhy1) and its *Arabidopsis* ortholog are quite alike in that these factors are absent from their respective promoters under repressing conditions and are drafted to their regulatory elements in the activation of the defense response (Desveaux et al., 2004; Boyle and Després, unpublished data). Although the Why1 is present at these loci under similar circumstances, the specific means by which this common factor contributes to the activation of the *PR-1* or that of the *PR-10a* has not yet been addressed.

A common theme that emerged through this study is the involvement of dual function factors in the regulation of *PR-1* and *PR-10a*. We demonstrated that the TGA2-clade of transcription factors are required for both the activation and repression of the *PR-1,* and we also showed that the known activator Pti4 is essential for the recruitment of the SEBF repressor to the *PR-10a*. It is rather surprising that one of the very few unifying features found in the regulation of these *PR* genes is the involvement of uncommonly treasonous transcription factors. While such behaviours are deemed unconventional, there are a number of factors that are known to deviate from their designations as activators

and repressors (See Chapter 2). The work in this thesis supports a functionally dynamic role for transcription factors in the regulation of gene expression.

6.3 Future Experiments

Future experiments should be aimed at addressing the contributions of the cis-acting DNA elements and DNA/chromatin structures to the regulation of *PR-1* and *PR-10a* expression, under both resting and inducing conditions. I would use the *in planta* transient expression system to introduce *PR-1* promoter mutants followed by antiTGA2 or antiNPR1 ChIPs to map which regions of the promoter are required for the recruitment of these regulators, under resting and activating conditions. Initially I would employ the LS promoter mutants used to address the *PR-1* cis-acting DNA regulatory elements (Lebel et al., 1998). If this strategy proves unfruitful I would resort to the use of 5' promoter deletions to determine the regions in which these regulators reside, and then narrow down the actual DNA elements required using LS mutations. To bolster the signal and specificity of these ChIP experiments the regulators in question could be transiently expressed bearing an epitope tag, along with the promoter deletions.

I would employ the bisulfite sequencing based method of Ronai et al. (2007) to map the regions of ssDNA within the *PR-10a* promoter. This technique enables the detection of ssDNA in vivo in the context of chromatin. These experiments would be conducted in both untreated and elicitor treated tubers to determine if and how *PR-10a* activation alters promoter DNA conformation. It would also be of interest to perform these experiments with SEBF and Pti4 knock downs to examine the specific contributions of these entities to ssDNA structures at the *PR-10a* promoter.

Chromatin architecture is a critical component of gene regulation. As mentioned previously, limited attempts to address the histone modifications involved in *PR-1* activation have proven inconclusive. I would conduct a comprehensive investigation of not only the major histone marks (See Chapter 2), but also the histones themselves present at the promoter under both non-inducing and activating conditions, using a ChIP strategy. Chromatin remodeling involving the eviction or displacement of promoter nucleosomes in gene activation events is well documented (Li et al., 2007). The work of Ng et al. (2006) used the ChIP technique to demonstrate a decrease in histone density at the promoter of a reporter gene upon transcription factor binding, *in planta*. A similar strategy could be used to investigate if chromatin architectural alterations are involved in the regulation of *PR-1* expression. This approach could also be employed to determine whether or not the *PR-10a* promoter is in anyway associated or occupied by nucleosomes.

Collectively the findings presented in this thesis have advanced our understanding of the mechanisms by which transcription factors, cofactors and promoter elements combine to control the expression of the *Arabidopsis PR-1* and potato *PR-10a* genes. While these genes are functionally related this project has established that the *PR-1* and *PR-10a* are governed by very distinct mechanisms. Such results would argue that the knowledge and understanding of plant immune response regulatory mechanisms, derived from the study of the model system *Arabidopsis*, does not trivially translate into crop species.

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